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This project concerned the synthesis of a series of peptidomimetics, eventually directed toward drugs that would interfere with angiogenesis and therefore act as potential anticancer agents. The peptidomimetics, based on the beta-turn motif, contained a dipeptide core, which maps onto the central two residues of the naturally occurring turn, and a linker portion. The linker is necessary to enforce the desired turn type and to provide room for a third amino acid side chain mimic. In the reporting period, a series of linkers based on phenylalanine was prepared and inserted into the cyclic peptidomimetics. Once prepared, the resulting macrocycles were shown to adopt predominantly type II or II' turns, depending on the location of the phenylalanine side chain.

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INTRODUCTION

This project concerned the synthesis of a series of peptidomimetics eventually directed toward drugs that would interfere with angiogenesis and therefore act as potential anticancer agents. The peptidomimetics, based on the beta-turn motif, contained a dipeptide core, which maps onto the central two residues of the naturally occurring turn, and a linker portion. The linker is necessary to enforce the desired turn type and to provide room for a third amino acid side chain mimic. In the reporting period, a series of linkers based on phenylalanine was prepared and inserted into the cyclic peptidomimetics. Once prepared, the resulting macrocycles were shown to adopt predominantly type II or II' turns, depending on the location of the phenylalanine side chain.

BODY

There were two PI's who worked during the grant period. Their contributions will be noted separately, followed by a section detailing the work accomplished to the aims set out in the original application.

Work accomplished by Mary MacDonald (first PI; 4/1/00 - 3/17/01). Previous work in the mentor's laboratory had established that macrocycles composed of a dipeptide unit and an aminocaproic acid (Aca) "linker" could function as β-turn equivalents. Since such compounds have been shown by other works to be important features in angiogenesis inhibitors,[1] this new paradigm of β-turn mimicry was directed toward the synthesis of compounds that would be useful in this manner. Most important angiogenesis inhibitors feature the so-called RGD motif, named for the three amino acids arginine, glycine, and aspartic acid, which seem to be essential for binding to the

biomolecular targets. To accommodate such targets into our peptidomimetic design, it was necessary to provide a place for a third amino-acid residue on the linker.

It was necessary to invent new synthetic protocols to accomplish this task. This work occupied nearly the entire yearlong period of the postdoctoral research fellowship. Specifically, synthetic routes to the peptidomimetics shown in Figure 1 were devised. The specific chemical routes to each compound are included in the manuscript provided in the appendix[2] and will not be reiterated here. The following general points, however, are germane: (1) the synthetic routes are flexible and will accommodate a number of amino acid side chains, (2) phenylalanine was used as a representative amino acid residue, and (3) the routes used a combination of novel synthetic technology and previously established chemistry. It is important to emphasize that the development of new synthetic routes such as these is a decidedly nontrivial undertaking.

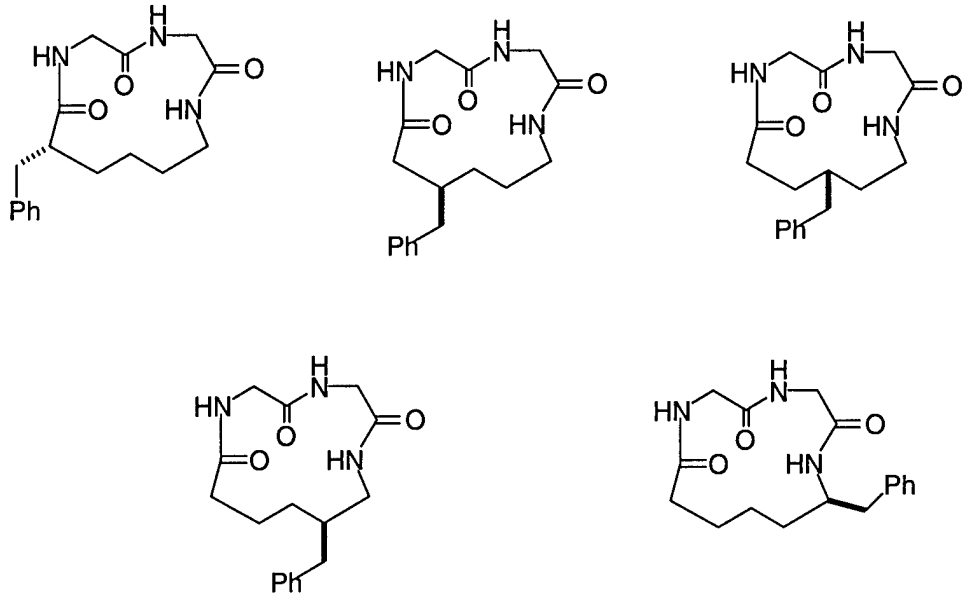

Figure 1. Peptidomimetics synthesized during the granting period. Synthetic routes are not shown.

In unpublished work, Dr. MacDonald was able to prepare groups in which the phenylmethyl substituents in Figure 1 were replaced by an allyl group as detailed in the

original proposal. However, there was insufficient time to convert these materials to the arginine and aspartic acid side chains during her tenure on the grant.

Once prepared, it was necessary to examine the solution phase conformations of these compounds to ensure that that in fact acted as mimics of β-turns. This was done using nuclear magnetic resonance and circular dichroism spectroscopies. It was ascertained that all but one of the substitution types either take up a type II turn or its mirror image type II' shape. The sole exception to this was the linker bearing the side chain on the central carbon (the upper right-hand compound in Figure 1). This is extremely encouraging for applications to vitronectin design. In addition, it was possible to obtain solid-state X-ray crystallographic for several of the compounds. Two such structures are shown in the Figure 2. These results completely verify our solution-phase data.

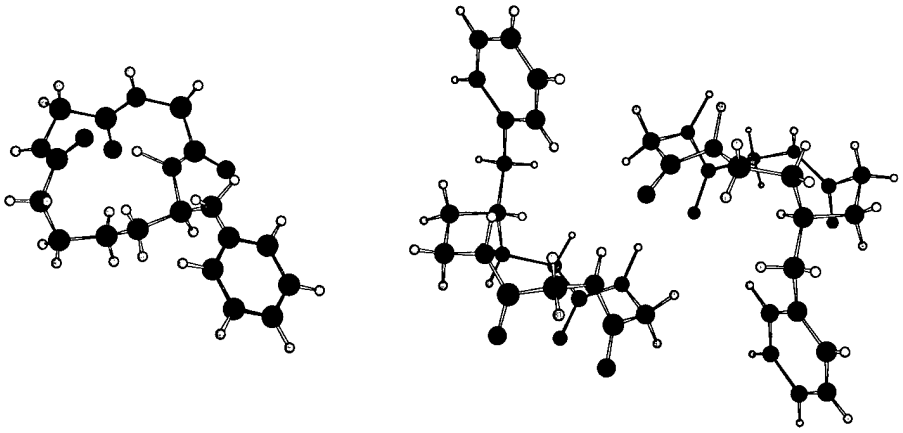

Figure 2. Representative X-ray structures of turns prepared.

Work accomplished by Christopher Katz (second PI; 3/18/01 - 4/30/01). One of the problems noted in the work described above was that a major synthetic technique used to prepared three of the five linker types needed was non-optimal. In the few months allotted to Mr. Katz on the granting period, he worked on a new protocol to prepare the

key intermediate in the overall scheme (Figure 3). This new methodology takes one synthetic step rather than the three needed by Dr. MacDonald and is significantly more efficient (for our purposes, intermediates A and A' are interchangeable). As the current grant has lapsed, this work will be completed out under a different funding mechanism.

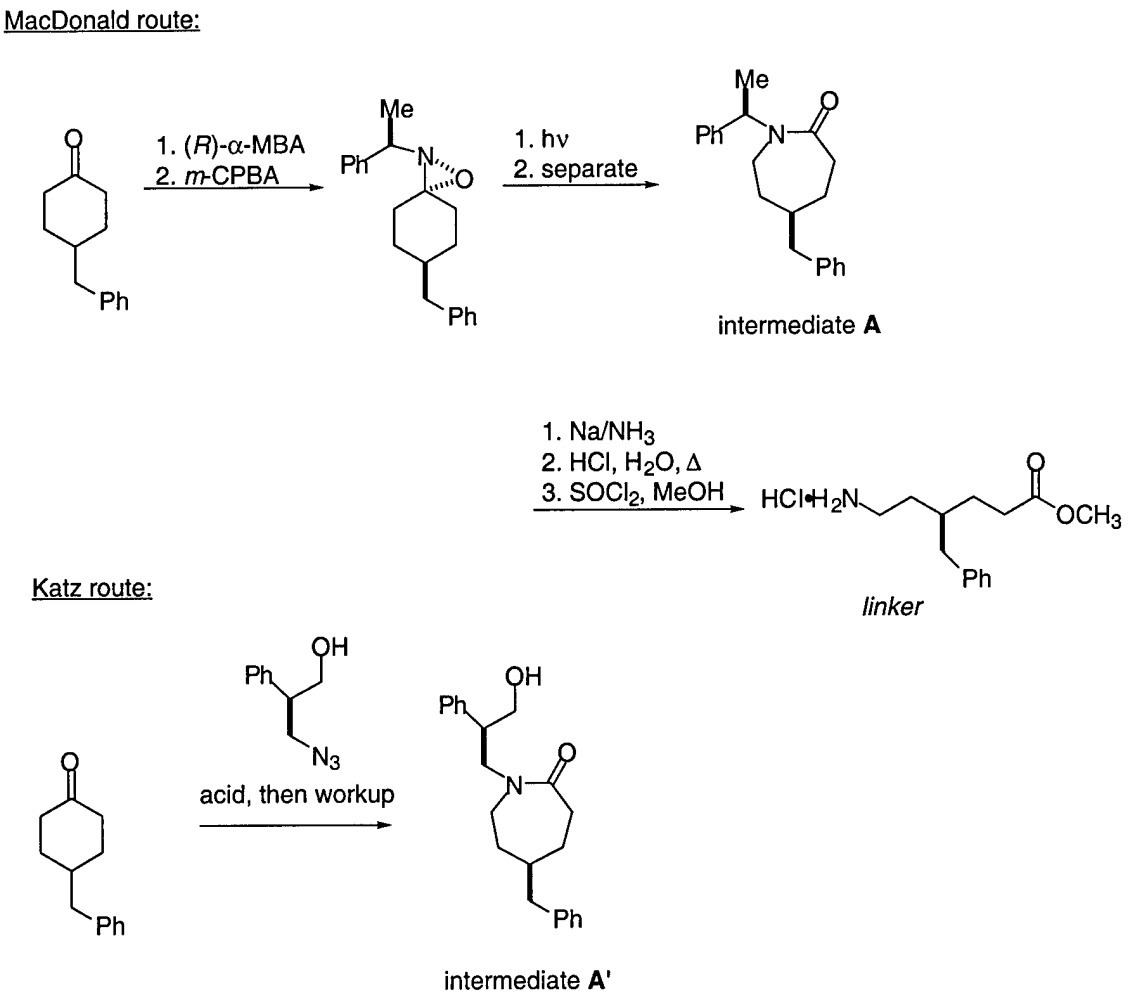

Figure 3. Comparison of synthetic routes enabling synthesis of intermediates A and A'.

Change of scope of the proposed work. There are two main considerations that had an impact on how the work was carried out. First, a change of PI from Mary MacDonald to Christopher Katz was approved on March 17, 2001. This change was requested due to the completion of Dr. Mary MacDonald's degree program, after which

time she left the University of Kansas to accept another position. The second issue pertains to the overall scope of this research program. The original statement of work as present in the reviewed proposal was for a two-year period and included the following tasks (taken directly from the original proposal):

> *Task 1.* To synthesize the first two generations of proposed vitronectin antagonists and perform a thorough conformational analysis using various spectroscopic methods to determine the β-turn type of the central dipeptide portion of the molecule (months 1-12).
>
> *Task 2.* To synthesize the third generation of compounds containing the extra methyl group within the peptide macrocycle, perform the same conformational analysis as mentioned above, and submit all prospective candidates for biological assays (months 13-24).

In actuality, the proposal was only funded for the 13-month period of 4/1/2000 - 4/30/2001. Accordingly, we were only able to complete a large portion of Task 1 during this period. The only significant part of her work left incomplete was the actual conversion of the allylated macrocycle analogs to arginine or aspartic acid mimics. Given the short time that Mr. Katz was on the proposal, it was decided that it would be more fruitful for him to shore up the existing sequences than to begin work on biological studies that he would not have time to complete in the funding period available.

KEY RESEARCH ACCOMPLISHMENTS

The key accomplishments during the funding period were:

- The development of versatile and efficient synthetic routes to five different classes of macrocyclic β-turn peptidomimetics.
- The solution- and solid-phase investigation of these classes of compounds, which verified that they indeed do take up the desired types of turn structures.
- The development of a streamlined route to the key intermediate A' (see Figure 3).

REPORTABLE OUTCOMES

Manuscript

Approaches to Cyclic Peptide β-Turn Mimics. MacDonald, M.; Aubé, J. *Curr. Org. Chem.* **2001**, *5*, 417-438.

Abstracts (presenter is underlined)

"Cyclic β-Turn Mimics Derived from Benzyl Aminocaproic Acids." Mary MacDonald, David Vander Velde, and Jeffrey Aubé, 219th National Meeting of the American Chemical Society, San Francisco, California, March 26-30, 2000, ORGN 799.

CONCLUSIONS

Although technical in nature, it is important to recognize that novel therapy is not possible without access to chemically novel agents. The work accomplished in this grant

period lays the foundation for a novel attack on the problem of integrin antagonism and the inhibition of angiogenesis. In a larger sense, the ability to mimic β-turns by merely plugging in various amino acids and linkers into the design described herein may also impact other areas of therapeutic significance.

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(1) Müller, G. *Angew. Chem., Int. Ed. Engl.* **1996**, *35*, 2767-2769.

(2) MacDonald, M.; Aubé, J. *Curr. Org. Chem* . **2001**, *5*, 417-438.

APPENDICES:

A reprint of reference (2) above follows.

Approaches to Cyclic Peptide β-Turn Mimics

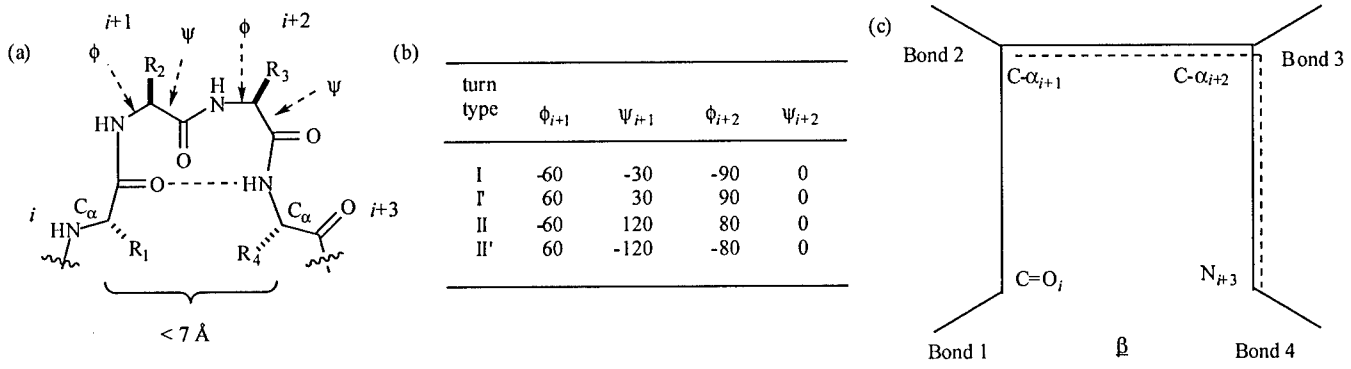
Mary MacDonald and Jeffrey Aubé*

Department of Medicinal Chemistry, University of Kansas, Lawrence, Kansas 66045-2506 USA

Abstract: The β-turn is a common recognition feature between peptide ligands and their macromolecular targets. The cyclization of a short peptide segment with a linker is one method of imitating this conformation. The first part of this review discusses tethering strategies which have resulted in the development of mimetics for the enkephalins and somatostatin as well as in the discovery of antagonists for targets such as thrombin, the CD4 protein on T lymphocytes, the integrins, and other receptors involved in inflammatory diseases. The second portion of this review describes the application of ε-aminocaproic acid (Aca) as a tether in cyclic peptide β-turn mimics. Alkyl substituents on Aca may influence the β-turn preference of the dipeptide. The synthesis of the substituted Aca linkers and their incorporation into the macrocycles is highlighted.

1. INTRODUCTION AND OVERVIEW

Peptides and proteins perform important functions in physiological systems ranging from biocatalytic processes to the transfer of information between cells. The amino acid sequence is not the only determinant of biological activity. Secondary structure is also important. Therefore, understanding the bioactive conformation of ligands as they are bound to their targets has been the impetus behind much of rational drug design. The β-turn, a nonrepeating motif, is the third most common secondary structure in proteins behind the α-helix and β-sheet. A β-turn is made up of four amino acids labeled *i*, *i*+1, *i*+2, and *i*+3 (Fig. (**1a,b**)). Up to eight different subtypes and their mirror images have been reported based on the dihedral angles, ϕ and ψ, of the *i*+1 and *i*+2 residues [1, 2]. The definition of the most common β-turns (types I, II, and their mirror images, I' and II') requires that the amide bonds reside in the trans orientation and that the distance between the α-carbon of the *i* and *i*+3 residues be < 7 Å [3]. Because the turn places the carbonyl of the *i* and the amide proton of the *i*+3 residue in close proximity, a hydrogen bond may exist between them, although it is not required. The likelihood of certain amino acids to participate in β-turns and the positions that they would preferentially occupy may be predicted using an algorithm based on the composition and placement of hundreds of known β-turn examples [4, 5]. This information has been used to predict the occurrence of similar bends in proteins according to the amino acid sequence.



turn type	ϕ_{i+1}	ψ_{i+1}	ϕ_{i+2}	ψ_{i+2}
I	-60	-30	-90	0
I'	60	30	90	0
II	-60	120	80	0
II'	60	-120	-80	0

Fig. (1). (a) Important features of a typical β-turn. (b) Idealized dihedral angles of the most common β-turn subtypes. (c) The definition of β, used in the simplified system [5].

β-Turn subtypes are traditionally defined by the geometry of the peptide backbone of the central dipeptide. Because there are variations or distortions of the dihedral angles in each subtype, an alternative system has been created to simplify the topographical definition of the turn unit [6]. This new system reduced the description of a β-turn to a single dihedral angle, β, consisting of two conformationally rigid units, one including bond 1, bond 2, and C-α_{i+2}, and the other including C-α_{i+1}, bond 3, and bond 4 (Fig. (**1c**)). This system places the emphasis on side chain orientation (e.g., bonds 2 and 3) rather than the dihedral angles of the central dipeptide since the amino acid side chains are

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important for interactions with the target. The creation of β was geared toward the design of peptidomimetics for which backbone geometry would no longer be relevant.

The conformation of peptides has been studied using a variety of techniques. The most widely used methods include nuclear magnetic resonance (NMR, particularly two-dimensional techniques such as NOESY, ROESY, and COSY), infrared (IR), and circular dichroism (CD) spectroscopies for solution-phase studies. X-ray crystallography provides information concerning the solid state conformation. The measurement of amide temperature coefficients (TCs) and solvent perturbation studies aid in determining which amide protons and carbonyl oxygens participate in hydrogen bonds. Because many compounds occupy various conformations in solution, computational methods such as conformational energy calculations and molecular dynamics provide information concerning conformer populations under different conditions.

We are primarily interested in short, tethered, cyclic peptides as β-turn mimics. While these will be covered in the second part of this review, other general approaches toward β-turn mimicry will be briefly considered here. One intention of β-turn peptidomimicry is to stabilize the relevant dihedral angles in the turn so they fall within the ranges appropriate for the sought-after turn type. Sometimes this can be accomplished by residue substitution in the parent peptide. For example, the use of *N*-methyl-, dehydro-, α-methyl-, or D-amino acids enhances the turn population in a peptide [7]. There are also a variety of methods that incorporate short-range cyclizations, which are called heterodetic cyclizations, into a peptide sequence by connecting amino acid side chains to form amide and ester linkages, or disulfide bridges. Each of these modifications has been observed in naturally occurring peptides. Other cyclizations that attach amino acid side chains to the peptide backbone are used in synthetic peptides and peptidomimetics. These strategies have been reviewed by Hruby[7] and Toniolo [8].

A well-known example of a short-range cyclization is the Freidinger lactam (Fig. (**2**)) [9]. As reported in one of the landmark papers of peptide mimicry, a lactam was embedded in the peptide backbone of a luteinizing hormone-releasing hormone (LHRH) analog by attaching a side chain to an amide nitrogen. The new analog showed greater potency than its parent hormone, which was attributed to a higher binding affinity for its receptor and increased metabolic stability. Structural variations, syntheses, and applications of Freidinger-like lactams have been extensively reviewed [10, 11].

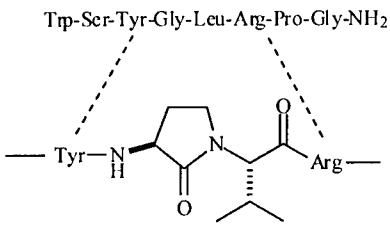


Fig. (2). Illustration of the Freidinger lactam substitution in LHRH.

As an aside, the β-turn conformation is a pertinent component of the β-hairpin, in which it constitutes the central portion of a strand-turn-strand motif. Based on a survey that β-turn types I' and II' are more prone than their mirror image to stabilize the β-hairpin conformation[12], Gellman and coworkers investigated the likelihood of various peptides to form hairpins and correlated these findings with the preferred β-turn conformation of the central dipeptide [13]. They did not assign turn types to the loop but found that the all L-amino acid sequences did little to induce hairpin formation. Inserting D residues in place of L residues, a method known to promote mirror image turn conformations, enhanced the amount of hairpin conformation. The propensity displayed by some of these peptides toward hairpin formation may result from the orientation of the hydrogen bonding moieties attached to the β-turn dipeptide. Thus, mirror image turn types may position attached peptide chains in closer proximity to form hydrogen bonds relative to their corresponding peptides containing all L-residues.

A common strategy for β-turn peptidomimicry is to replace the *i*+1 and *i*+2 residues of the turn with a scaffold to stabilize the turn structure. This type of scaffold best serves its purpose if (1) it provides sites for attaching side chain-like appendages, (2) it positions an amide bond similar to that in the central dipeptide of the β-turn, and (3) it possesses amine and carboxylic acid groups so the scaffold may be incorporated into a peptide chain in place of the putative turn residues. The majority of these turn mimics are bicyclic and tricyclic heterocycles. Many syntheses of these peptidomimetics have been made amenable to solid-phase techniques and combinatorial library construction, as is the case for the template in Fig. (**3**) [14]. A variety of these cyclic turn motifs have been reviewed by Hanessian and Lubell [11].

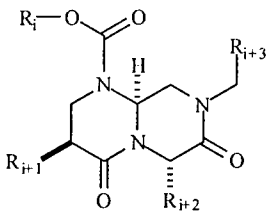


Fig. (3). A β-Turn peptidomimetic designed for solid phase synthesis.

Another form of β-turn mimic replaces the $i \rightarrow i+3$ hydrogen bond with a covalent linkage to form a ten-membered ring. For example, the templates shown in Fig. (**4**) represent macrocycles in which a dipeptide-like backbone has been constrained with a covalent bond to resemble a β-turn conformation. This motif in Fig (**4a**) was used in the design and synthesis of a conformationally restricted peptidomimetic designed to imitate a β-turn in the CD4 protein found mainly on the surface of T lymphocytes [15]. This system was later modified by inserting alkyl or alkenyl amines in place of X with the hope of developing enkephalin mimics (Fig. (**4b**)) [16].

β-Turns are a common recognition feature between ligands and receptors, providing many targets for peptidomimetic design. These mimics have been useful as

both agonists and antagonists. The biological targets will be mentioned throughout with specific examples. However, many of the applications are focused on the design of integrin antagonists [17]. The integrins are a family of cell surface receptors which are involved in cell-cell and cell-matrix interactions. The sequence Arg-Gly-Asp (RGD) has been identified as a recognition sequence in several of the ligands for the various integrins. Accordingly, many integrin antagonists are designed to mimic the RGD motif. The most commonly studied integrins are the fibrinogen ($\alpha_{IIb}\beta_3$) and vitronectin ($\alpha_v\beta_3$) receptors. Activation of $\alpha_{IIb}\beta_3$ leads to platelet aggregation, so antagonists of this integrin may be useful in dissolving blood clots. Alternatively, the $\alpha_v\beta_3$ integrin is involved in a variety of physiological outcomes that include bone regeneration, acute renal failure, and tumor metastasis. It functions largely as a mediator of angiogenesis, which is the process leading to the formation of new blood vessels. Based on X-ray structures obtained for fibronectin, an RGD-dependent ligand, the amino acid triad participates in a type II' β-turn, thus providing a starting point for antagonist design [18, 19].

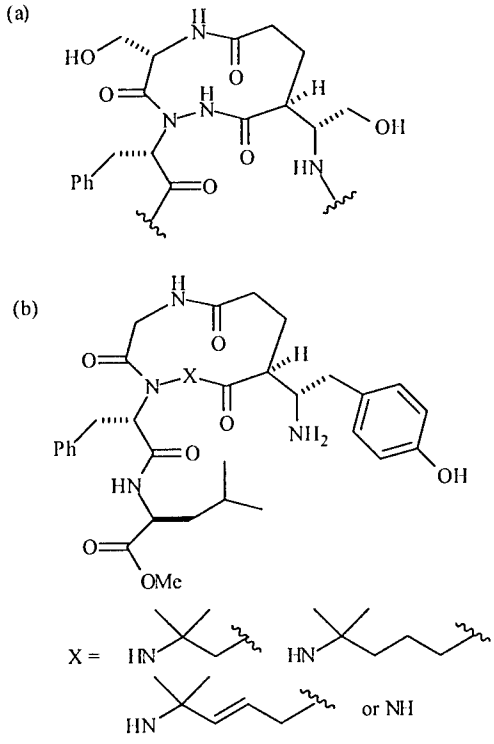


Fig. (4). β-Turn mimics incorporating hydrogen bond replacements designed for (a) mimicking the CD4 protein on T lymphocytes and (b) evaluation as enkephalin peptidomimetics.

2. PEPTIDE MACROCYCLES

Peptide cyclization can constrain a short amino acid sequence into a turn conformation. In contrast to the heterodetic cyclizations described above, the coupling of the *N*- and *C*-termini of a peptide is called a homodetic cyclization. In particular, five- and six-residue cyclic peptides have served as models for reverse turns (Fig. (**5**)). In general, cyclic hexapeptides can adopt conformations containing two β-turns, often referred to as the turn-extended-turn conformation [17]. In contrast, it has been established that pentapeptides like *cyclo*(Gly-Pro-Gly-D-Ala-Pro) can adopt a conformation containing both β and γ turns. In solution, this particular peptide resides predominantly in a βII/γ turn conformation in a variety of solvent and temperature combinations as determined by NMR and CD [20]. An X-ray crystal structure supported these findings in the solid state [21]. Variations in the peptide sequence influenced the turn type, causing mixtures of types I and II conformations in the β-turn region. While there are many examples of β-turns in cyclic peptides, this review will focus on those that mimic RGD.

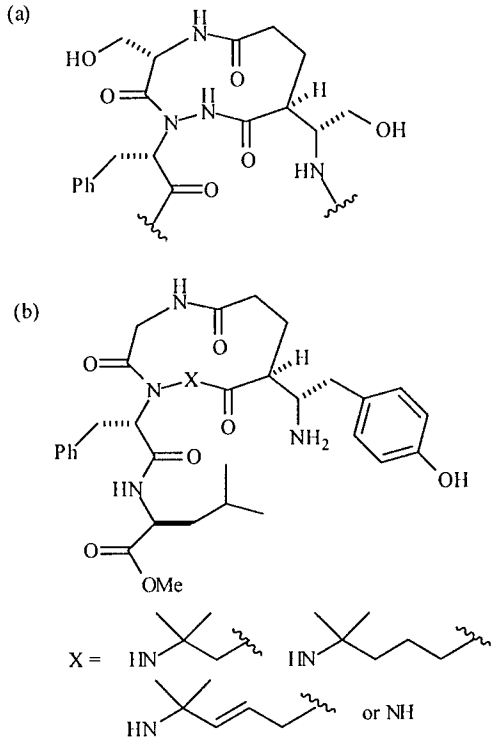
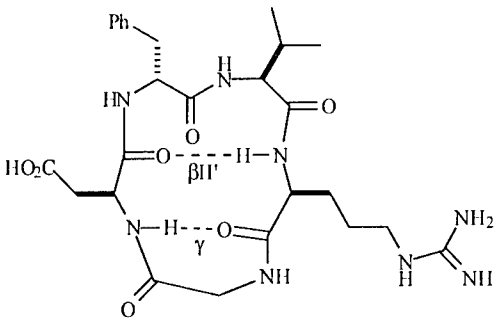


Fig. (5). A cyclic pentapeptide containing both a β-turn and a γ-turn.

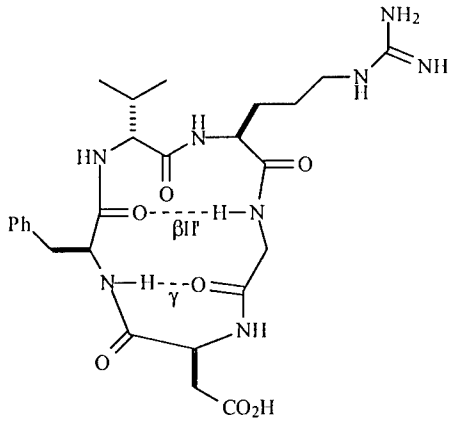
Cyclic Peptides

It was found that cyclic pentapeptides containing the RGD triad prevented binding of natural protein ligands to $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ better than the linear analogs [22]. The incorporation of D-amino acids into the sequence produced the two peptides, *cyclo*(Arg-Gly-Asp-D-Phe-Val) and *cyclo*(Arg-Gly-Asp-Phe-D-Val), which best inhibited the binding of vitronectin or the laminin fragment P1 to $\alpha_v\beta_3$. Similar to earlier reports on cyclic pentapeptides, these structures contained both a type II' β-turn and a γ-turn (Fig. (**6**)). In both cases, the D-amino acid occupied the *i*+1 position of the β-turn, with the major difference between the two peptides being the position of the RGD sequence relative to the β-turn. This conformational difference affected the orientation and distance between the arginine and aspartic acid side chains, which were found to be important in antagonist selectivity. In the case of *cyclo*(Arg-Gly-Asp-D-

Phe-Val) and *cyclo*(Arg-Gly-Asp-Phe-D-Val), the former prevented the binding of both vitronectin and laminin fragment P1 to the $\alpha_v\beta_3$ receptor. On the other hand, *cyclo*(Arg-Gly-Asp-Phe-D-Val) was more selective and prevented the binding of laminin fragment P1 better than vitronectin. The decreased selectivity of *cyclo*(Arg-Gly-Asp-D-Phe-Val) was ascribed to a conformational change within the peptide causing it to adopt a conformation similar to *cyclo*(Arg-Gly-Asp-Phe-D-Val) [23].



cyclo(Arg-Gly-Asp-D-Phe-Val)

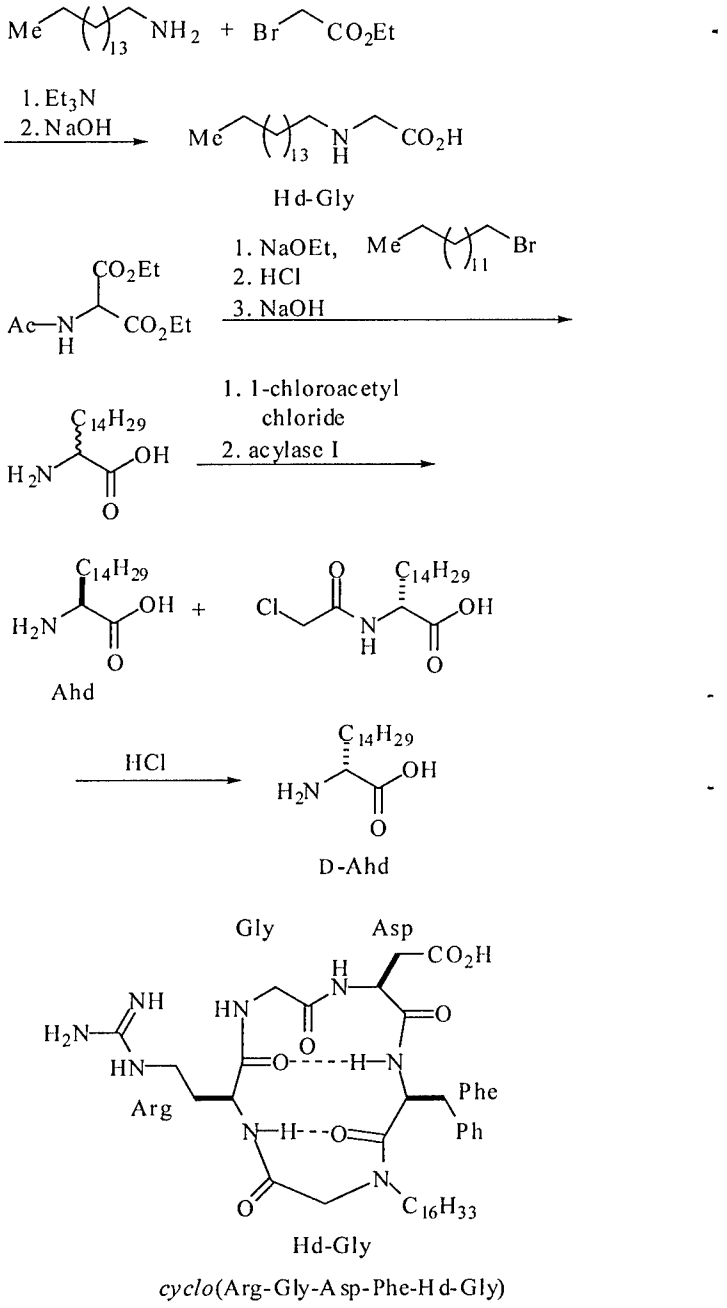


cyclo(Arg-Gly-Asp-Phe-D-Val)

Fig. (6). Cyclic pentapeptides containing RGD.

Burgess et al. investigated the effects of cyclic $(RGD)_2$ against $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ [24]. Surprisingly, the conformation of *cyclo*(RGDRGD) resembled the earlier example of *cyclo*(Arg-Gly-Asp-D-Phe-Val) rather than one typical of a cyclic hexapeptide. Analysis showed that both had a γ-turn centered around a glycine residue. The cyclic dimer, however, contained a βI conformation centered about the aspartic acid and arginine residues rather than a βII' turn. The hexapeptide also demonstrated selectivity for binding to $\alpha_v\beta_3$ over $\alpha_{IIb}\beta_3$, but the acyclic RGDRGD standard was a better inhibitor, suggesting that cyclization of the hexapeptide probably constrained the backbone in a less than optimal conformation for $\alpha_v\beta_3$ binding.

Efforts to improve the pharmacokinetics of cyclic pentapeptides have been made by incorporating unnatural, lipophilic amino acids. Thus, *N*-hexadecylglycine (Hd-Gly) and (2*S*)- and (2*R*)-2-aminohexadecanoic acid (L-Ahd and D-Ahd, respectively), were introduced into Kessler's cyclic pentapeptides in place of either the phenylalanine or valine residues [25]. Scheme 1 shows the synthesis of Hd-Gly, which began by treating ethyl bromoacetate with hexadecylamine. Saponification of the ester yielded Hd-Gly. The synthesis of (±)-Ahd began with treatment of diethyl acetamidomalonate with 1-bromotetradecane/NaOEt followed by hydrolysis and decarboxylation. The mixture was acylated with 1-chloroacetyl chloride and then resolved enzymatically using acylase I from *Aspergillus mellius* to produce (2*S*)-Ahd and (2*R*)-*N*-chloroacetyl-Ahd. The R enantiomer (D-Ahd) was obtained following treatment of the chloroacetyl derivative with HCl. Some inhibitory activity was seen with *cyclo*(Arg-Gly-Asp-Phe-Hd-Gly) against $\alpha_v\beta_3$. Conformational analysis showed that the macrocycle took on a βII'/γ motif with glycine in the *i*+1 position of the β-turn. However, a majority of the lipoderivatized compounds showed no biological activity when assayed for their ability



cyclo(Arg-Gly-Asp-Phe-Hd-Gly)

Scheme 1.

to inhibit the binding of biotinylated fibrinogen and vitronectin to $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$.

Natural and Synthetic Peptide Replacements

In a variation of the cyclization strategy, up to three amino acids in a cyclic peptide may be replaced with a tether. These replacements include long-chain amino acids, cyclic scaffolds, and other small molecules containing the functionality for incorporation into a peptide. These replacements have been used to examine the role of the certain residues in the peptide and the effect of the linker on the preferred turn conformation within the peptide.

The long-chain amino acid, δ-aminovaleric acid (Ava), was incorporated into the cyclic octapeptide, gramicidin S (GS), to investigate the contribution of various amide bonds to its antimicrobial activity (Fig. (7)) [26]. Ava was chosen because it resembles a Gly-Gly dipeptide minus the central amide bond. It is comparable in length to a dipeptide when both are fully extended. A series of GS analogs in which Ava replaced different dipeptide segments was subjected to antimicrobial assays and conformational analysis using CD and optical rotatory dispersion (ORD). Compounds with a single Ava substitution showed decreased biological activities, while the CD displayed patterns similar to the parent peptide but with decreased intensities. On the other hand, analogs containing two Ava substitutions had no activity, and the spectra resembled those for a random structure. It was concluded that (1) the conformation of the cyclic structure relied on a type II β-turn centered at Phe-Pro [26], (2) the amide proton between Pro-Val was a hydrogen bond donor [27], and (3) the Leu side chain was probably necessary for activity [28].

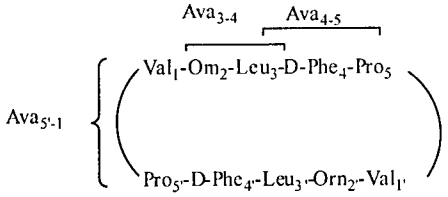
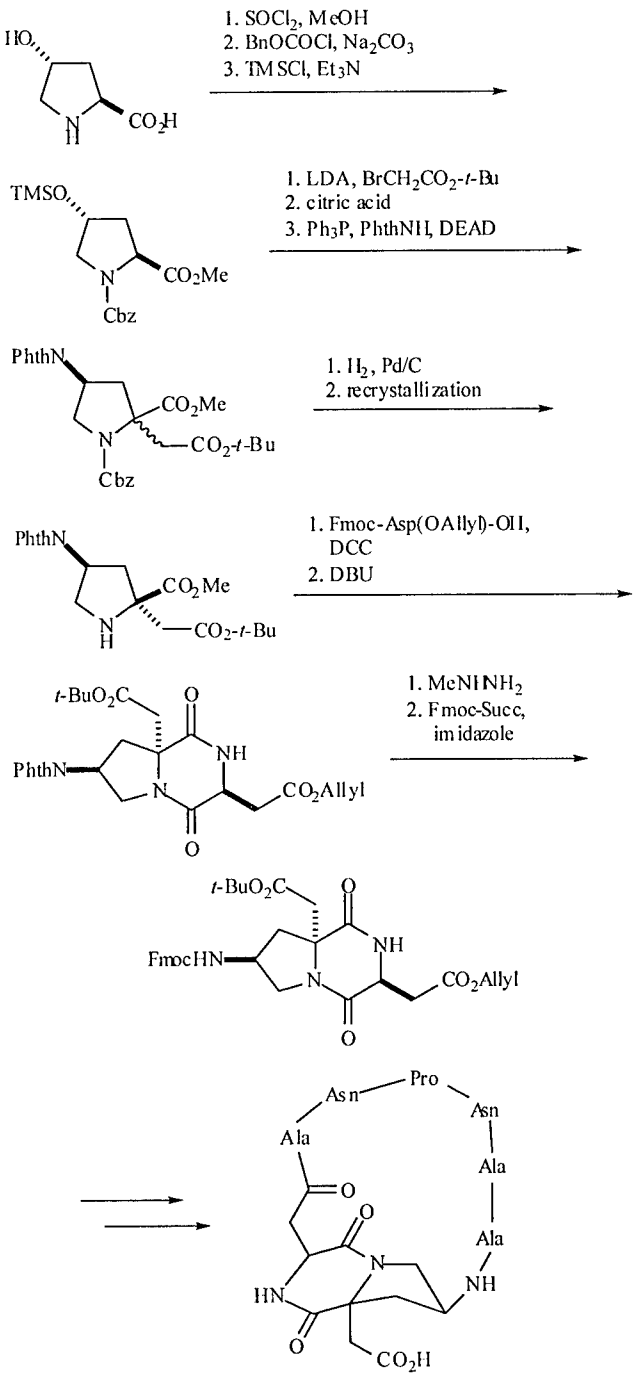


Fig. (7). Ava substitutions in GS.

Ava was also used to replace a single glycine residue in the peptide Boc-Leu-Val-Val-D-Pro-Gly-Leu-Val-OMe, which was known to form a β-turn around D-Pro-Gly [29]. Replacement of glycine with Ava was expected to extend the turn region to roughly three residues in length. However, comparison of NMR and CD data of the parent peptide and its analog revealed almost identical behavior. Molecular dynamics simulations using NOE-derived restraints produced two families of structures, differing only in the conformation of the Ava residue.

In addition to their utility as replacements for *i*+1 and *i*+2 amino acids of a β-turn in acyclic peptides, bicyclic heterocycles have also been used to tie peptides into a loop [30]. In research directed toward the development of an antimalarial vaccine, Asn-Pro-Asn-Ala (NPNA, a repetitive sequence found in a protein believed to be involved in the transference of malaria) was predicted to exist in a βI turn. Efforts toward raising antibodies against this protein and examining their protective effects against infection in rodents were successful, but application of this strategy in humans has been elusive. It was hypothesized that cyclization of the sequence Ala-NPNA-Ala using a bicyclic heterocycle would stabilize the βI conformation and aid in the promotion of antibody generation.

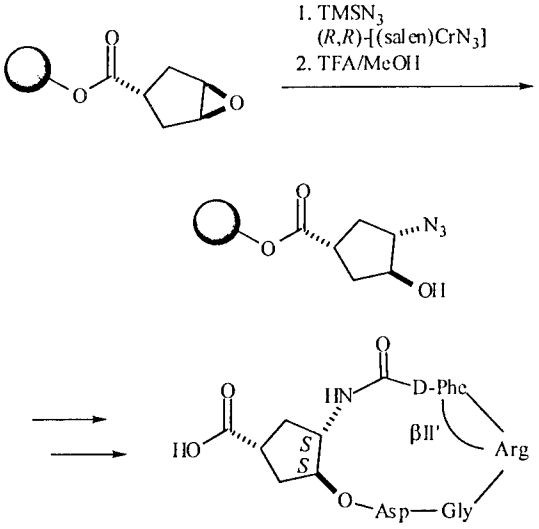
The bicyclic scaffold was derived from (4*R*)-hydroxyproline (Scheme 2). After protection of each of the functional groups, the α-center was alkylated with *tert*-butyl bromoacetate. The protected amine was introduced via a Mitsunobu reaction on the hydroxyl group freed by



Scheme 2.

deprotection using acid. After hydrogenolysis of the Cbz group, the requisite diastereomer was obtained by recrystallization. A peptide coupling to Fmoc-Asp(OAllyl)-OH, followed by intramolecular cyclization, formed the diketopiperazine. Manipulation of the protecting groups furnished the linker, which was incorporated into the peptide using solid-phase peptide synthesis. A detailed NMR analysis confirmed the presence of a βI bend. Mice immunized with a multiple-antigen peptide including the tethered hexapeptide produced antibodies against the target protein, thus supporting the presence of a βI turn in the NPNA region. Similar proline-based heterocycles have also been used in the formation of β-sheets [31].

In a study originally aimed at examining stereoselective transformations on a solid support, a new linker was developed and incorporated into cyclic RGD-containing peptides [32]. A support-bound cyclopentane *meso*-epoxide was subjected to an asymmetric ring-opening with trimethylsilyl azide, as catalyzed by a [(salen)CrN_3] complex (Scheme 3). The resulting polymer-bound azido alcohol was then available for further manipulation and derivatization. Diastereomers of the cyclopentyl azido alcohol were incorporated into cyclic pentapeptide-like molecules. Conformational analysis of the series of diastereomeric macrocycles indicated that the stereochemistry of the linker influenced the β-turn preferences within the peptide. For instance, the cyclic peptide containing the S,S-configured tether had a type II' β-turn, whereas the R,R diastereomer had a type I conformation. Both macrocycles containing the cis tethers had a γ-turn at the glycine residue, but the arginine and aspartic acid side chains had different orientations with respect to the arginine and aspartic acid side chains. Comparison of the affinities for $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ showed that the substrates all had a preference for the latter but with different degrees of selectivity.

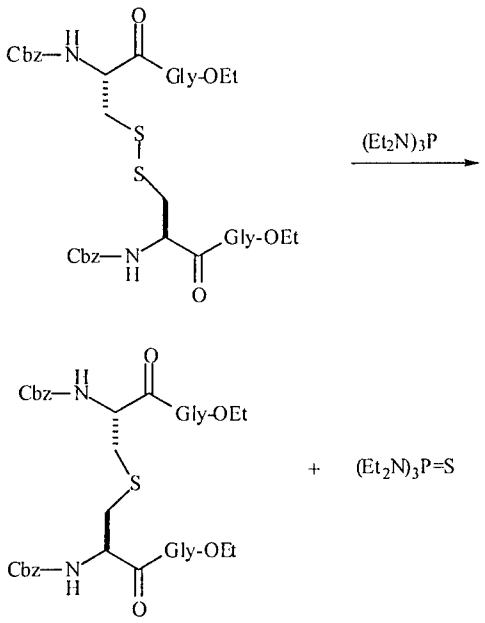


Scheme 3.

Linked Di- and Tripeptides

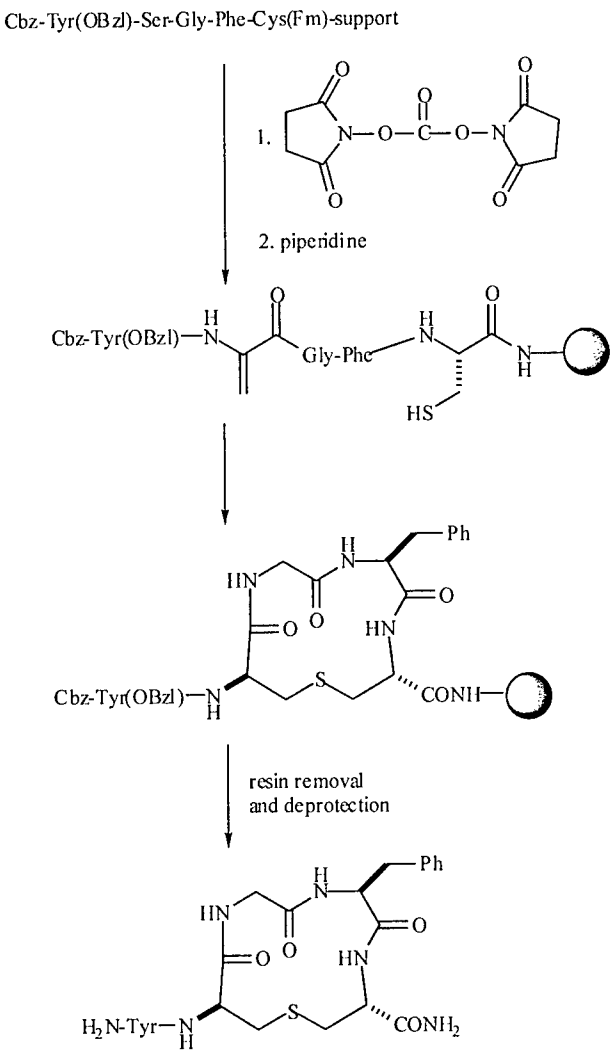
Most relevant to our own efforts, short peptides cyclized with a synthetic or nonpeptide tether can adopt β-turn conformations. A variety of peptide tethers differing in length and structure have been reported. These tethers restrict the number of possible conformations available to the macrocycle without distorting the peptide bonds into the cis orientation.

Lanthionine was a logical choice for such a tether. It is a naturally occurring amino acid resembling two alanine residues joined at the side chains by a thioether linkage. Thus, it is a monosulfide analog of cystine and is more stable to enzymatic degradation. Early efforts to establish the lanthionine linkage within a peptide involved the treatment of a disulfide with tris(diethylamino)phosphine to form the corresponding monosulfide with retention of configuration at the α-centers of the participating amino acids (Scheme 4) [33].



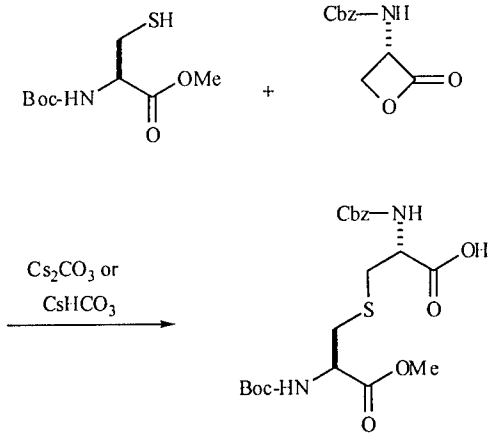
Scheme 4.

Lanthionine has also been used in the design of cyclic enkephalin mimics [34]. At the outset, it was known that the natural ligand adopted a βI turn-like conformation in the Gly-Pro region when bound to the μ receptor [35]. The bioactivity of cyclic enkephalin analogs containing a disulfide bridge connecting two D-penicillamine (D-β,β-dimethylcysteine) residues had been reported [36]. The formation of a lanthionine derivative of this peptide entailed the synthesis of the peptide precursor on a solid support, dehydration of the serine side chain using disuccinimidyl carbonate to form dehydroalanine, and exposure to piperidine to remove the fluorenyl methyl (Fm) group from the cysteine thiol (Scheme 5). Ring closure proceeded via a Michael addition of the cysteine thiol to the dehydroalanine. The analog was isolated after deprotection and removal of the solid support. Although Michael additions are not always stereoselective, only one diastereomer prevailed. The stereochemistry of lanthionine in the cyclic peptide was determined to be H_2N-Tyr-*cyclo*(D-Ala_L-Gly-Phe-Ala_L) when compared to peptide analogs containing diastereomeric lanthionine residues. Lanthionine is abbreviated as Ala_L and D-Ala_L. These were synthesized according to the protocol shown in Scheme 4.



Scheme 5.

A stereoselective route was developed to generate orthogonally protected lanthionine by nucleophilic attack of the thiolate from Boc-(*S*)-cysteine methyl ester on the serine-derived β-lactone, *N*-Cbz-(*S*)-3-amino-2-oxetanone (Scheme 6) [37]. Use of the cesium salt of the thiolate helped to suppress formation of the thioester and increase yields of



Scheme 6.

lanthionine. Replacement of cysteine with a more sterically hindered nucleophile, such as penicillamine, increased the regioselectivity of the reaction leading to the desired attack at the β carbon of the lactone. This result, which may be due to steric interactions between the substituted β carbon and the lactone carbonyl, directly produced protected lanthionine analogs useful in peptidomimetic synthesis.

Smaller rigid nonpeptide molecules may also serve as linkers; an important example of this is *meta*-(aminomethyl)benzoic acid (Mamb) [38]. The ability of Mamb to restrict peptide conformation was examined in a study aimed at developing more potent and selective integrin antagonists. Examination of a number of cyclic peptides revealed a degree of conformational flexibility, which was believed to affect, in part, their stability and oral activity. Efforts to design a more conformationally stable molecule began with an inspection of the pliancy of tethers or functional groups. The replacement of a disulfide bridge or a dipeptide in a cyclic peptide with Mamb significantly reduced the flexibility of the overall peptide macrocycle [38]. A structure-activity relationship study was carried out, in which tetrapeptides constrained with Mamb were assayed for their ability to inhibit binding of fibrinogen to the $\alpha_{IIb}\beta_3$ receptor [39]. This study showed that *cyclo*(D-Xaa-*N*-methylArg-Gly-Asp-Mamb), where Xaa = aminobutyric acid (Abu) or valine, were the most effective compounds (Fig. (**8**)). According to CD, Mamb constrained each peptide into a βII' turn. These new analogs exhibited higher binding affinities and selectivities for the $\alpha_{IIb}\beta_3$ receptor as well as increased metabolic stability over the most potent compound known at the time, SK&F 106760, which contains a disulfide bridge resulting from the coupling of cysteine and penicillamine side chains [40].

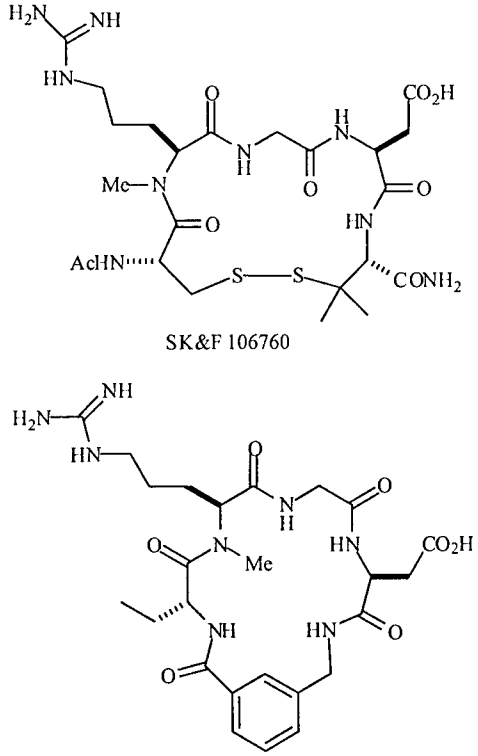
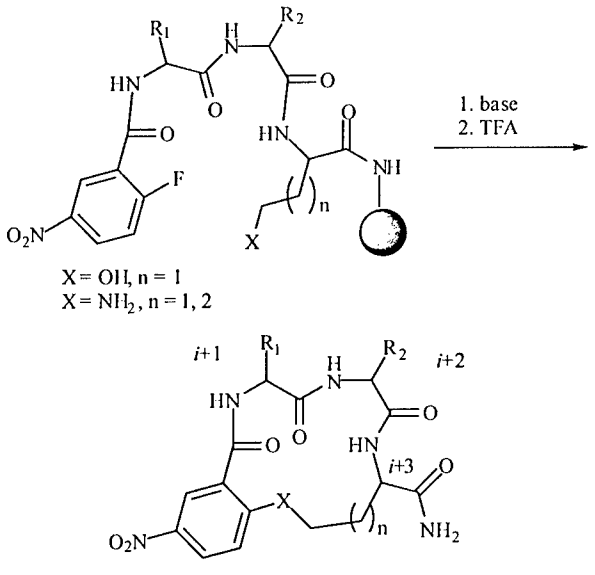


Fig. (8). Constrained peptides used to inhibit the integrin, $\alpha_{IIb}\beta_3$.

Both $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ utilize RGD-containing ligands for activation, leading to a question as to which RGD conformation is necessary to activate each receptor. Examination of the solid-phase and solution-phase conformations of synthetic ligands selective for either $\alpha_{IIb}\beta_3$ or $\alpha_v\beta_3$ has helped to uncover the conformational requirements of RGD-containing macrocycles. *cyclo*(D-Abu-*N*-methylArg-Gly-Asp-Mamb), with a type II' β-turn centered on the D-Abu-*N*-methylArg dipeptide, displayed selectivity for the $\alpha_{IIb}\beta_3$ receptor. Exchanging these residues with L,L-Ala-Arg resulted in a strong selectivity favoring the $\alpha_v\beta_3$ receptor and a type I β-turn for the analogous residues. Thus, a conformational change in at least this particular turn mimetic appears to accompany a change in receptor specificity [41].

A system reported by the Burgess group utilizes a tether composed of a substituted benzoic acid attached to the *N*-terminus of a tripeptide. Macrocyclic ring closure was effected by a base-promoted S_NAr coupling between the aryl group and the homoserine side chain of the *C*-terminal amino acid (Scheme 7). The compounds were synthesized on a solid support that was removed after ring closure. The S_NAr coupling proceeded in high yield and formed fairly structured macrocycles as indicated by NMR studies. Molecular dynamics simulations and amide TCs revealed that the amide bond of the *i*+3 residue participated in a hydrogen bond with the carbonyl oxygen of the *N*-terminal acyl group to form the ten-membered ring typical of β-turns [42].

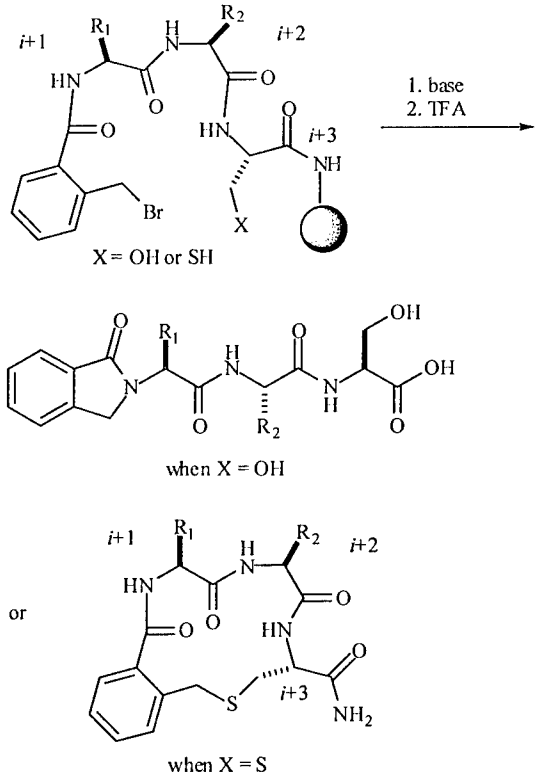
This system was further examined to determine how variations in the stereochemistry and tether affected the conformations of a series of nerve growth factor (NGF) analogs, which would be used to probe the NGF binding region (Scheme 7) [43]. By systematically substituting D- for the L-amino acids at each position, CD revealed that the stereochemistry at the *i*+2 position had the most influence on the β-turn type. The macrocycle preferred a βI turn was preferred with the L-amino acid in the *i*+2 position and a βII turn with a D-residue in the same position. Otherwise, changes in the tether heteroatom (e.g., from oxygen to nitrogen) and in the length of the *i*+3 side chain did not alter the macrocycle in its conformational preference.



compound	*i*+1	*i*+2	*i*+3	turn type
a	L	L	L	I
b	L	D	L	II
c	D	L	L	I
d	L	L	D	I

Scheme 7.

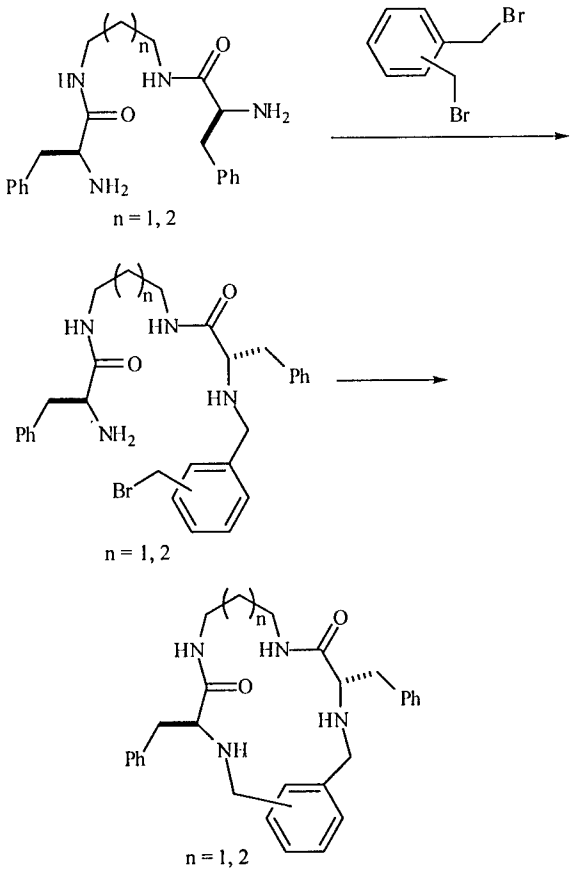
The replacement of the S_NAr reaction with an S_N2 displacement to close the macrocycle facilitated the synthesis of a new series of compounds (Scheme 8) [44]. Initially, serine was chosen as the *i*+3 residue since its nucleophilic hydroxyl group could, in principle, displace the bromide on the substituted *N*-terminal aryl ring. Instead of macrocyclization, however, base treatment resulted in *i*+1 amide substitution leading to the *N*-terminal benzolactam acyclic peptide shown. When cysteine was used in place of serine, the S_N2 ring closure was successful. In general, these compounds were more flexible than the S_NAr-based library, showing characteristics of both type I and II β-turns.



Scheme 8.

It has been suggested that cyclization substrates may be preorganized into β-turn-like conformations prior to ring closure, thus favoring intramolecular over intermolecular processes [45]. In the example shown in Scheme 9, precursors derived from amino acids and diamines of varying carbon chain lengths (n = 1, 2, and 3) were treated with *ortho*-, *meta*- and *para*-bis(bromomethyl)benzenes to form an acyclic precursor. A second nucleophilic displacement of bromide with a terminal amine produced the

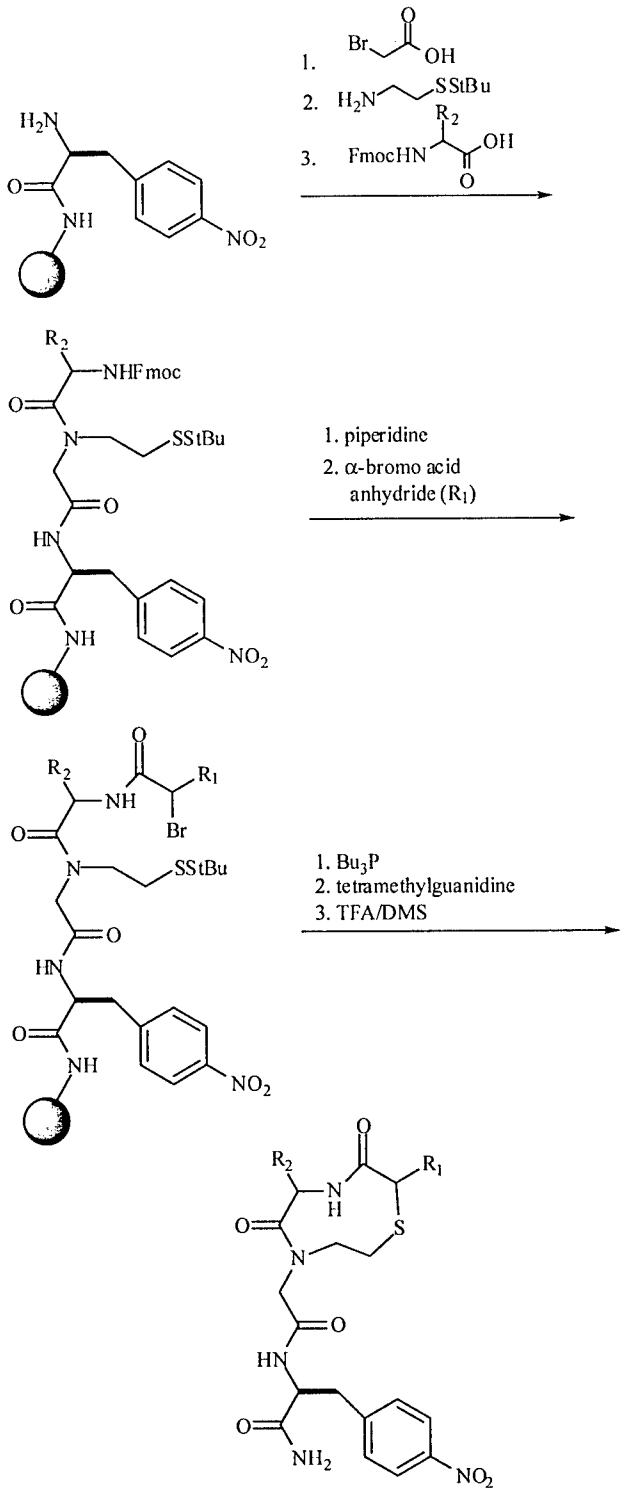
polyazacyclophane. The products from the *meta*- and *para*-bis(bromomethyl)benzenes were isolated in reasonable yield, whereas compounds containing the *ortho* isomers did not afford macrocyclic products. Molecular dynamics calculations indicated shorter distances between the nucleophilic nitrogen and the *meta*- and *para*-bromomethyl groups in the acyclic molecules prior to cyclization, whereas the *ortho* analogs favored an unfolded conformation. It was suggested that preorganization led to the superior results obtained in the *meta*- and *para*-series.



Scheme 9.

The increasing popularity of solid-phase organic chemistry has facilitated the creation of large β-turn libraries. A β-turn can be dissected into components, making it amenable to diversification. As mentioned earlier, correlating the biological activity of each analog with its structure and conformation may provide information regarding the three-dimensional requirements of the target.

An example of a turn mimic developed for such a function is shown in Scheme 10 [46]. The components include an α-halo acid to contribute the *i*+1 side chain and an α-amino acid to provide the *i*+2 residue. The linker to close the macrocycle was supplied by either 2-aminoethanethiol *tert*-butyl disulfide or its propane homolog. Thus, the coupling of α-bromoacetic acid to the polymer-bound *para*-nitrophenylalanine, an S_N2 displacement of the bromide with the amine from the future linker, and a second peptide coupling of an α-amino acid (*i*+2 surrogate) furnished the protected polymer-bound tripeptide. Deprotection and treatment with an α-bromo acid anhydride (*i*+1 side chain) completed the installation of the *i*+1 and *i*+2 side chains. Reduction of the disulfide using tributylphosphine produced the substrate for cyclization, which was completed upon treatment with tetramethylguanidine. The *para*-nitrophenylalanine was retained after the resin was removed to act as a chromophore to determine the purity of the final product. The entire synthesis consisted of eight steps and afforded crude products that were approximately 75% pure by HPLC.



Scheme 10.

Determination of β-turn content was carried out on a similar template minus the *para*-nitrophenylalanine residue while using methyl groups as both the *i*+1 and *i*+2 side chains set in the R and S configurations, respectively. A conformational search analysis using NMR-derived constraints produced low energy conformers that best resembled a βII' turn.

Alterations to this mimetic rendered a structure that also included the *i*+3 side chain as shown in Fig. (**9**). Libraries of this type later provided compounds to (1) mimic somatostatin [47, 48], (2) block activation of the *N*-formyl-Met-Leu-Phe receptor, which is involved in inflammation [49], and (3) block the activation of $\alpha_4\beta_1$, an integrin also involved in the inflammatory response [50].

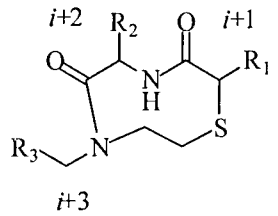


Fig. (9). Template for a β-turn mimic library that includes the *i*+3 side chain.

Rigid spacer molecules have also been effective in separating the *N*- and *C*-terminal residues at just the right distances to position the peptide in a β-turn conformation (Fig. (**10**)). For example, Albert and Feigel demonstrated how subtle changes in a steroidal pseudo-amino acid (Spa) could alter the turn conformation of a dipeptide in a dimeric macrocycle [51]. Three analogs of Spa were prepared from the methyl esters of 7-deoxycholic acid and cholic acid, differing only in the presence or absence of methyl esters on the steroid scaffold. Each analog was coupled to Boc-Phe-Phe-OH and then dimerized to form *cyclo*(Spa-Phe-Phe-Spa-Phe-Phe) analogs. Investigation of feasible conformations using molecular dynamics revealed the possibility of four conformations, two γ loop structures, a β-turn, and a turn resembling that found in an α-helix. Amide TC measurements and NMR data provided evidence that two of the structures contained a large percentage of the β-turn conformation in the dipeptide backbone. In contrast, the third compound appeared to be more flexible. The conformational data indicated the existence of an equilibrium between two conformations. It was postulated that this type of cyclic structure could act as a cavity suitable for the encapsulation of organic guests.

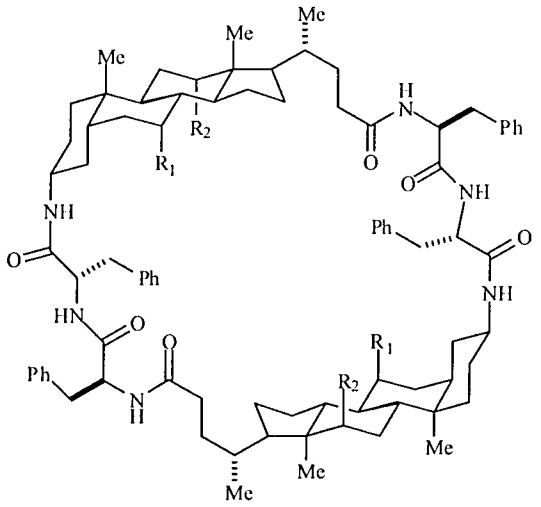


Fig. (10). Dimeric Spa-Phe-Phe analogs form different conformations depending on Spa substitution.

3. THE ACA TETHER

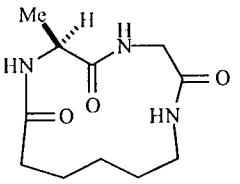
As mentioned in the introduction, cyclic β-turn mimics can be envisioned by either replacing the pertinent dipeptide in the turn or by retaining the dipeptide and cyclizing it with a constraint. The examples described in the previous section portrayed β-turn mimics synthesized according to the latter method. ε-Aminocaproic acid (Aca) has been successfully used to constrain dipeptides into β-turn mimics. This section covers background relevant to the development of these molecules and the results of our studies of Aca-constrained dipeptides.

The Aca linker was first carefully investigated in peptides by Scheraga, Woody, and coworkers, who sought to establish spectroscopic parameters for β-turns in peptides and proteins [52-55]. Because open-chain peptides are flexible and exist as a mixture of conformations in solution, small, cyclic peptides seemed to be an ideal model system. Based on the criteria that (1) the *i*+1 and *i*+2 residues should not be in an α-helical conformation, and (2) the distance between the α-centers of the *i* and *i*+3 residues should be < 7 Å, Aca was deemed the best candidate to act as the "third" amino acid in a cyclic tripeptide. Therefore, *cyclo*(Ala-Gly-Aca) was chosen as the model peptide (Fig. (**11**)). It included the requisite *i*+1 and *i*+2 residues derived from natural amino acids and the three amide bonds present in a β-turn. It was important that the ω-amino acid not be so short as to prevent conformational fluctuations in the peptide macrocycle. When fully extended, the distance between the Aca C-α and C-ε carbons was 5.04 Å, well below the 7 Å limit. Aca was short enough to restrict the number of possible conformations allowed to the tethered dipeptide, yet it would not distort the amide bonds into the cis orientation, according to MM2 calculations. It was determined that a βII turn was the favored conformer for *cyclo*(Ala-Gly-Aca), along with contributions from up to 35% of the type I conformation. The NMR data indicated that the macrocycle was conformationally biased. A low TC for the Aca amide proton suggested that it participated in a hydrogen bond between the Aca amide and the Aca carbonyl, which would be the equivalent of the $i \rightarrow i+3$ hydrogen bond in standard β-bends. NOE data supported the presence of a type II turn, because a crosspeak was seen between the Ala_{α} and Gly_{NH} protons. Crosspeaks between Ala_{NH} and Gly_{NH}, which are typical for βI conformations, were not evident.

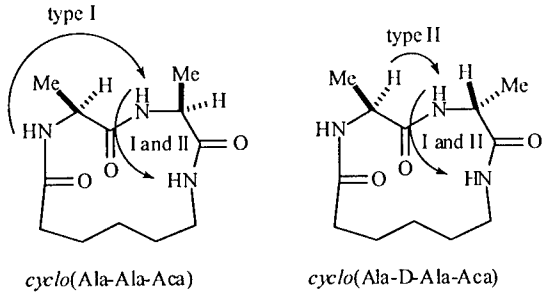
Inspection of *cyclo*(Ala-Gly-Aca) using IR and Raman spectroscopies provided information about its conformation

in the solution and solid states [54, 56]. The amide stretching region is a diagnostic tool used to examine hydrogen bonding. The IR spectrum of *cyclo*(Ala-Gly-Aca) supported the existence of a type II β-turn as the major conformation with two weak hydrogen bonds, one between Gly_{NH} and $Aca_{C=O}$ then, etc. following an adjustment of dihedral angles, between Aca_{NH} and $Aca_{C=O}$. There was, however, a minor contribution of βI in aqueous solution. When dissolved in $CHCl_3$, βI stretches were not evident. The solid-phase data was wholly consistent with a βII bend.

Similar analyses of *cyclo*(Ala-Ala-Aca) and *cyclo*(Ala-D-Ala-Aca) indicated a predominance of type I and II β-turns, respectively (Fig. (**11**)) [52]. Spectroscopic data for the latter greatly resembled the data obtained for *cyclo*(Ala-Gly-Aca), verifying a βII conformation. The L,L analog appeared to be prone to association in solution. Predictions of low energy conformations for the L,L showed both Ala_{NH} protons pointing in the same direction, thus providing hydrogen bond donors for the Aca and Ala-1 carbonyl groups of a neighboring molecule. The NMR signals also showed some averaging which could be the result of either association or greater conformational flexibility, but the former was deemed more likely.



cyclo(Ala-Gly-Aca)



cyclo(Ala-Ala-Aca) *cyclo*(Ala-D-Ala-Aca)

Fig. (11). Illustrations of the macrocycles used by Scheraga, Woody, and coworkers. The notations depict the standard NOEs observed in types I and II β-turns.

CD has been a powerful tool in the determination of peptide and protein secondary structure. Since peptides are flexible in solution, spectral analysis of β-turn types were previously determined by correlating data from CD, NMR, and molecular dynamics [57]. The development of the Aca-cyclized β-turn mimics provided more conformationally biased templates for CD analysis [52, 53]. The macrocycles are often referenced as standards for type I and II β-turns [58]. More recently, Fasman, Perczel, and coworkers formulated algorithms based on the NMR and CD data from groups of similar β-turn-containing compounds to deconvolute the CD spectra of samples prone to adopting multiple conformations [59, 60].

3,5-Dimethyl Aca

Work in these laboratories has centered on the effects of Aca substitution and its stereochemistry on the β-turn preference of Aca-linked dipeptides. Several interrelated hypotheses formed the basis of this work. Carefully placed substituents could twist Aca, thereby affecting the peptide backbone. Substituents could affect the physical properties of the macrocycle (e.g., hydrophilicity and hydrophobicity) and provide additional functional groups to interact with a biological target. Finally, they could also act as points of attachment for solid supports.

The first set of Aca linkers examined comprised all of the diastereomers of 3,5-dimethyl Aca. This substitution pattern was chosen based on the well-known importance of *syn*-pentane interactions in controlling conformation [61]. A series of studies by Hoffmann and coworkers have examined these principles in the context of acyclic conformational control. For example, the substitution pattern in 2,4-dimethylpentane causes the flexible hydrocarbon backbone to adopt mainly two conformations in a 1:1 ratio (Fig. (**12a**)). Different groups in the 2- and 4-positions influence the conformations formed by the hydrocarbon backbone in order to minimize unfavorable steric and electronic interactions [62]. This effect was evident in the conformations adopted by 2-hydroxy-4-vinylpentane, which was found to exist as an **a:b** ratio of 3.5:1 as a result of decreased *gauche* interactions between the vinyl group and the proton attached to the tertiary carbon adjacent to the hydroxyl group (Fig. (**12b**)). The effect of substitution on longer carbon chains was also examined. Although the molecule shown in Fig. (**12c**) has five rotatable backbone linkages, only one conformation

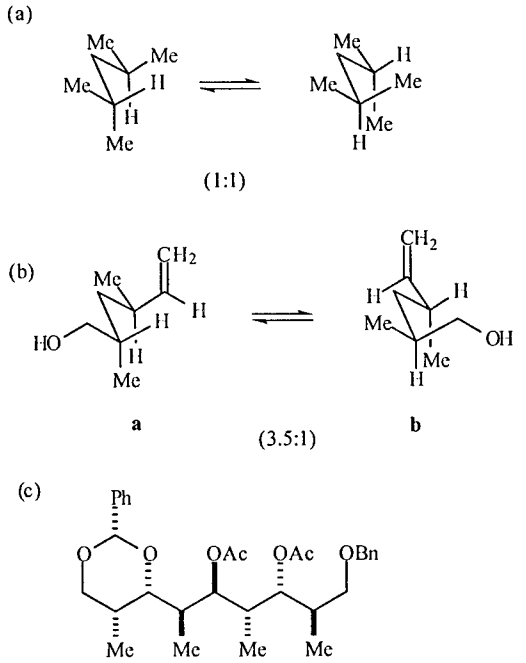


Fig. (12). (a) 2,4-Dimethylpentane adopts two conformations of equal energy. (b) This pentane analog prefers one conformation over the other. (c) A substituted alkyl chain with 5 rotatable backbone bonds that mainly adopts a single conformation.

prevails [63]. These studies were designed to provide some insight into the conformational preferences of longer substituted hydrocarbons, because similar patterns are common in many polyketide natural products.

This concept was implemented in the design of a β-turn mimic [64]. A dimer of 2-vinyl-4-hydroxymethylpentane containing an *N*-acetyl and a dimethylamide at each end yielded a bent hydrocarbon (Fig. (**13**)). This conformationally biased carbon chain could be superimposed on the backbone of a β-bend, in which the *trans*-olefin is a bioisostere for the amide bond between the *i*+1 and *i*+2 residues. Spectroscopic analysis showed that the molecule resided mainly in one conformation. IR revealed a stretch at 3360 cm^{-1}, which is one characteristic of a hydrogen-bonded amide proton. The IR spectrum of an unsubstituted analog showed both hydrogen-bonded and non-hydrogen-bonded amide proton stretches. Finally, analysis of an analog with methyl groups in place of the carboxamide groups proved that the structured conformation was not solely the result of hydrogen bonding but was partly due to the minimization of *syn*-pentane interactions.

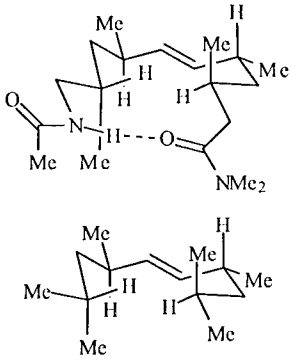
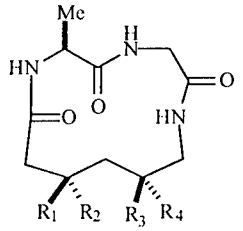


Fig. (13). Both a bis(amide) and its all carbon analog adopt a β-turn-like conformation.

We chose to examine the moderately flexible dipeptide Ala-Gly cyclized with all four stereoisomers of 3,5-dimethyl Aca pictured in Fig. (**14**) [65]. The substituted tethers were obtained following a series of transformations previously developed in this laboratory [66, 67]. The key step was the

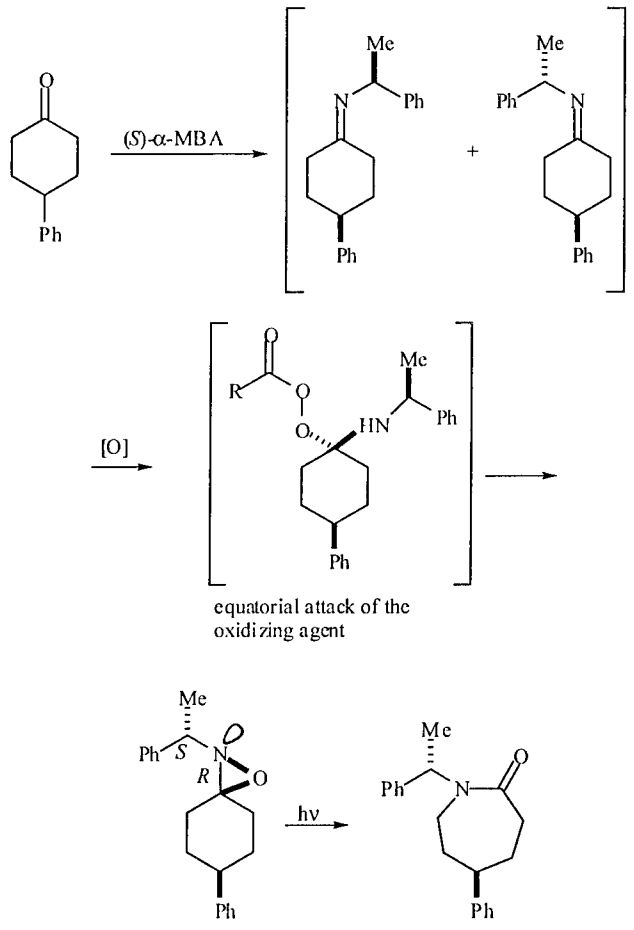


	R_1	R_2	R_3	R_4
1	H	Me	H	Me
2	Me	H	Me	H
3	Me	H	H	Me
4	H	Me	Me	H

Fig. (14). A series of macrocycles in which Ala-Gly is cyclized with 3,5-dimethyl Aca.

formation of an oxaziridine mixture which underwent a stereospecific ring expansion to form the corresponding caprolactam (Scheme 10) [68]. Group selective transformations like this have attracted significant attention over the past decade [69, 70]. However, as we began this project, this oxaziridine-mediated ring expansion was the only practical way to carry out a group-selective nitrogen-insertion reaction. Since then, we have recorded progress on a potentially powerful new technique that uses chiral hydroxy azides, but this work is beyond the scope of the present review [71].

The stereochemical aspects of the oxaziridine formation and the ring expansion have been reviewed, so only the salient aspects will be summarized here [67]. Synthesis of the oxaziridines began with the appropriately substituted cyclohexanone (Scheme 11). The starting cyclohexanone was condensed with enantiomerically pure α-methylbenzylamine (α-MBA). The resulting imine was oxidized in situ with *meta*-chloroperoxybenzoic acid (*m*-CPBA) to form the corresponding oxaziridine. The orientation of the oxaziridine ring in the product placed the nitrogen and oxygen atoms in the axial and equatorial positions of the spiro-fused six-membered ring, respectively. This suggested that the oxidant approached the equatorial face of the imine, which is anchored by the C-4 substituent present in the initial cyclohexanone. Addition of the peracid to the imine resulted



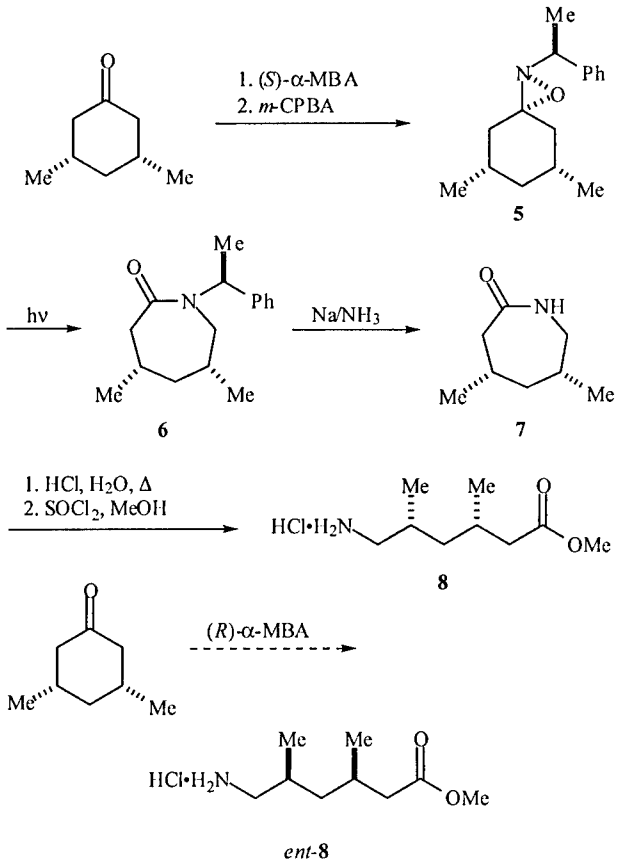
Scheme 11.

in a tetrahedral intermediate, permitting rotation about the carbon-nitrogen bond. After ring closure, the major diastereomeric oxaziridine possessed the *unlike* stereochemistry between the benzylic carbon of α-MBA and the oxaziridine nitrogen according to the Cahn-Ingold-Prelog descriptors.

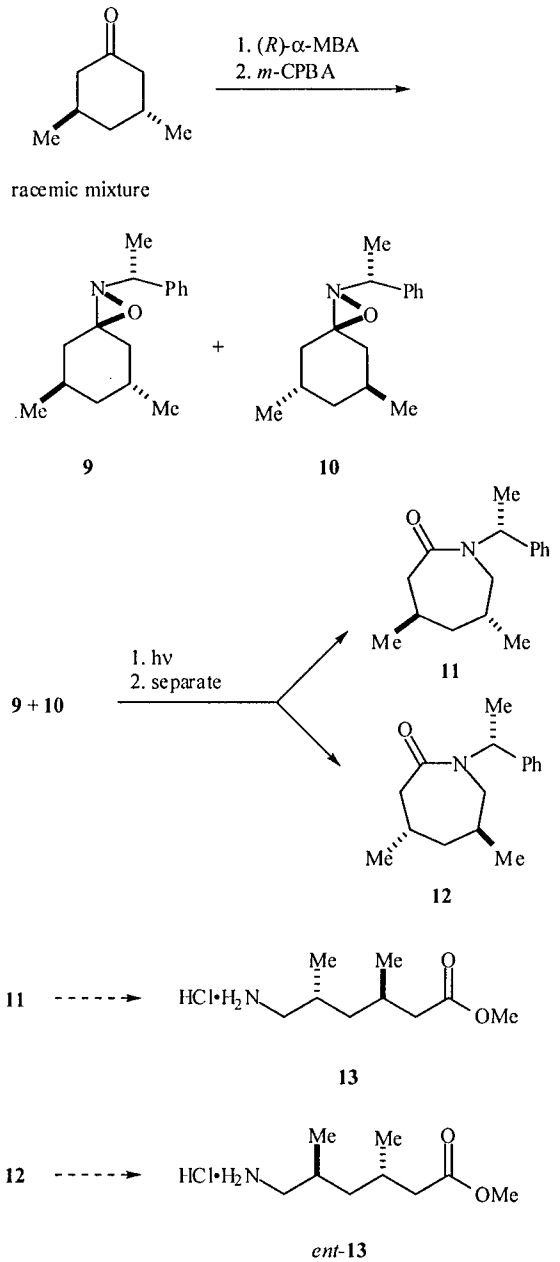
The photochemical rearrangement of oxaziridines to form the corresponding lactams had been reported by Lattes and coworkers [72]. In examples of oxaziridines derived from substituted cyclohexanones, they found that ring expansion yielded mainly one of two regioisomeric lactams and suspected that the stereochemistry of the oxaziridine nitrogen played an important role in the selectivity. Overall, caprolactam formation requires the cleavage of a carbon-carbon bond followed by the formation of a carbon-nitrogen bond. Although the mechanism of this reaction remains unclear, it has been established that the regiochemistry of the photochemical ring-expansion step depends on the configuration at the oxaziridine nitrogen [68]. The major product in all examples recorded to date results from cleavage of the carbon-carbon bond antiperiplanar to the lone pair of electrons on nitrogen. In this way, control of oxaziridine stereochemistry can lead to a regioselective ring expansion process.

Thus, the *cis*-3,5-dimethyl Aca tethers were synthesized starting with *cis*-3,5-dimethylcyclohexanone. Condensation with (*S*)-α-MBA to form the imine followed by oxidation with *m*-CPBA rendered a mixture of oxaziridines with **5** as the major diastereomer (Scheme 12). Photolysis of the oxaziridine mixture yielded a mixture of *N*-phenethyl lactams, which were separated by column chromatography. The major diastereomer **6** was treated with Na/NH_3 to reductively remove the phenethyl group, yielding **7**. Acid hydrolysis of the lactam followed by esterification produced the hydrochloride salt of *cis*-3,5-dimethyl Aca methyl ester **8**. Its enantiomer, *ent*-**8**, was synthesized simply by replacing (*S*)-α-MBA with (*R*)-α-MBA.

The *trans*-3,5-dimethyl Aca enantiomers were synthesized beginning with *trans*-(±)-3,5-dimethylcyclohexanone (Scheme 13). Although the enantiomerically pure ketones would directly afford a single non-racemic lactam due to their C_2-symmetry, practical considerations suggested that it would be easier to begin with racemic ketone and carry out a combination ring-expansion/resolution maneuver. Thus, racemic starting material afforded a mixture of two major diastereomeric



Scheme 12.

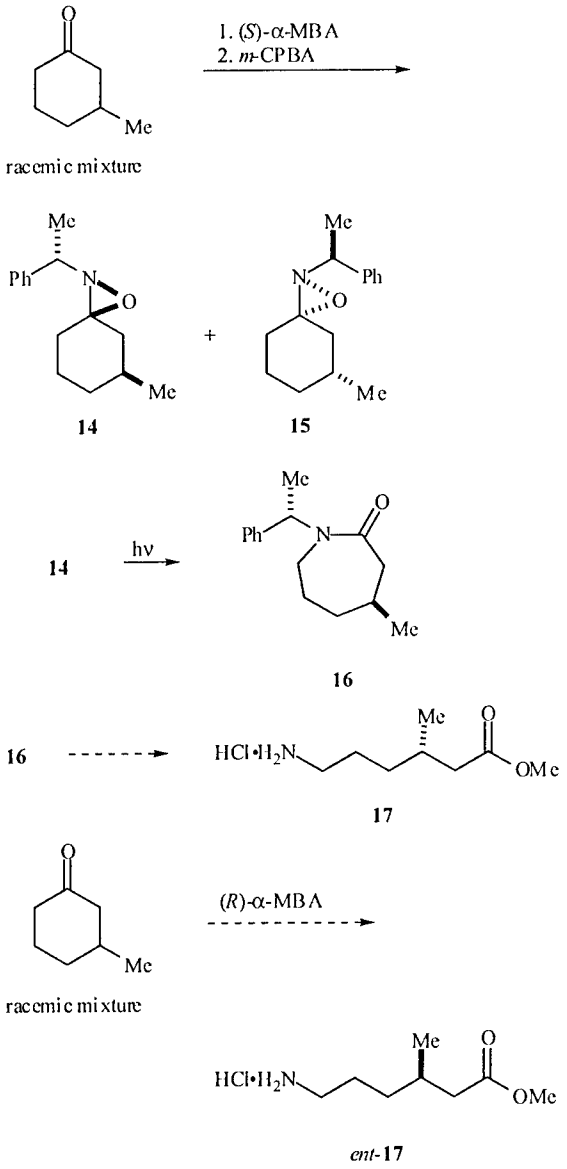


Scheme 13.

oxaziridines, **9** and **10**, in a 1:1 ratio. The mixture was photolyzed to produce the corresponding *N*-phenethylcaprolactams **11** and **12**, which were then separated by column chromatography. Each lactam underwent a Birch reduction to remove the phenethyl group, and acidic hydrolysis followed by an esterification to produce each enantiomer of the *trans*-3,5-dimethyl Aca methyl ester **13** and *ent*-**13** as its hydrochloride salt.

Preliminary efforts toward examining the effects of a monomethyl Aca tether were also begun. Based on the conformational analysis of the macrocycles using 3,5-dimethyl Aca, it was suspected that substitution at the C-3 position played an important role in the conformational preferences of the ring. Therefore, (3*R*)- and (3*S*)-methyl Aca were synthesized for use in the macrocycle syntheses.

The use of an unsymmetrical ketone brings additional regiochemical aspects to the oxaziridine-forming reaction. A 3-substituted cyclohexanone could produce a 3- or a 5-substituted Aca tether. The regioselectivity is a combination

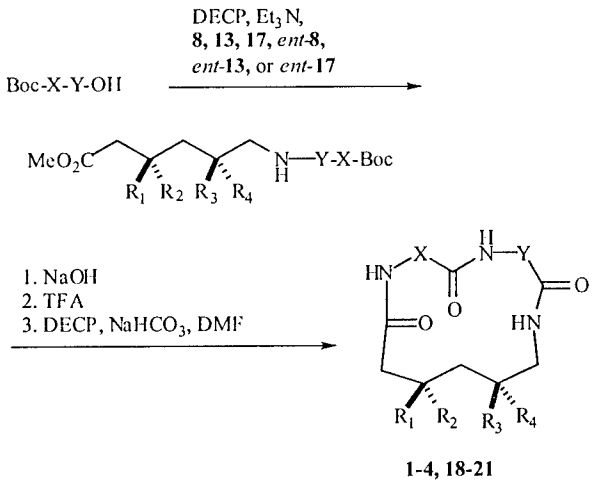


Scheme 14.

of the stereochemistry of the starting ketone and the stereoisomer of the α-MBA used. Therefore, the combination of a racemic ketone and enantiomerically pure α-MBA would eventually furnish both the 3- and 5-substituted Aca tethers.

Condensation of (±)-3-methylcyclohexanone with (*S*)-α-MBA afforded diastereomeric oxaziridines **14** and **15** (Scheme 14). Diastereomer **14** was separated from the mixture by column chromatography then photolyzed to form the nitrogen–insertion product **16**. Thus, **17** was obtained following the reaction sequence described earlier. The use of (*R*)-α-MBA in place of the S enantiomer in the oxaziridine synthesis yielded *ent*-**17**.

The tethers were coupled to Boc-Ala-Gly-OH, Boc-Gly-Gly-OH, or Boc-Ala-Ala-OH using diethylcyanophosphonate [73] (DECP) to form the tripeptide (Scheme 15). After saponification of the ester and trifluoroacetic acid (TFA) deprotection of the Boc group, the tripeptide was cyclized using DECP under dilute conditions. It was worth noting that the cyclization yields for the more highly substituted compounds were much higher (51-71%) than the yield for the macrocycle containing a single methyl group in the ring (35%).



Scheme 15.

Conformational analysis of the macrocycles was carried out using NMR and CD spectroscopies. The NMR studies were conducted in DMSO-d_6 because the water solubility of the compounds was very low. However, incremental addition of D_2O to the samples did not result in any noticeable changes in the NMR spectra, suggesting that the DMSO conformations may be similar to those in aqueous solutions. Obviously, this point will need to be further investigated using macrocycles with greater water solubility. COSY and ROESY spectra revealed cyclic structures with predominantly one conformation. Two-dimensional NMR studies indicated that compounds **2** and **3** each had interactions between the Ala_{α} and Gly_{NH} protons, which was indicative of a type II β-turn. Interactions between Ala_{NH} and Gly_{NH}, typical of the type I β-turn, were not evident in **3** and could not be determined in the case of **2** since the signals were not as well resolved. This latter NOE

Table 1. Diagram for the Synthesis of Cyclic Peptides

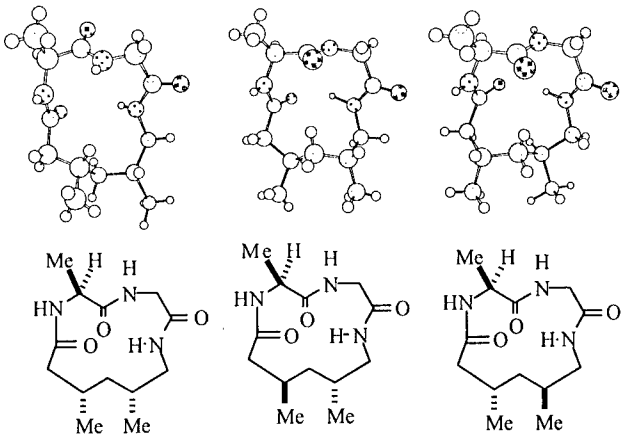
entry	linker	X-Y	R_1	R_2	R_3	R_4	cyclic peptide
a	**8**	Ala-Gly	H	Me	H	Me	**1**
b	*ent*-**8**	Ala-Gly	Me	H	Me	H	**2**
c	**13**	Ala-Gly	Me	H	H	Me	**3**
d	*ent*-**13**	Ala-Gly	H	Me	Me	H	**4**
e	**17**	Ala-Gly	H	Me	H	H	**18**
f	*ent*-**17**	Ala-Gly	Me	H	H	H	**19**
g	*ent*-**17**	Gly-Gly	Me	H	H	H	**20**
h	**13**	Ala-Ala	Me	H	H	Me	**21**

was visible, however, in **1** along with a crosspeak at Ala_{α}-Gly_{NH}. All of the macrocycles in this first series also showed interactions between Gly_{NH}-Aca_{NH}, which is common to both βI and βII turns.

Analysis by CD was done in methanol (c = 1 mg/mL). Compounds **2** and **3** best resembled the standard, *cyclo*(Ala-Gly-Aca), which is known to exist mostly as a βII turn. Each showed a maximum and a minimum ellipticity at ca. 203 nm and 220 nm, respectively. The curve for **4** had the same maximum but lost the well-defined minimum near 220 nm. On the other hand, the CD spectrum of **1** resembled that of a type I turn with some type II present. Based on this CD data, it appeared that the (3*S*)-methyl group in the disubstituted Aca linkers redirected the conformation from a strong type II β-turn toward a mixture of types I and II. To better define the relationship between the stereochemistry at C-3 and the macrocyclic conformation, the (3*R*) and (3*S*)-monomethyl tethers were incorporated into the cyclic peptides. Thus, **18** possessed the 3*S* configuration and, like the other examples possessing the S stereochemistry, lacked the distinct minimum at 230 nm by CD. The 3*R* epimer, **19**, was similar to **2** and **3**.

To investigate the conformational effects of a single substitution in the entire macrocycle, **20** was synthesized. This example does not contain any side chains in the dipeptide region to influence the β-turn preference. Therefore, the major conformational determinant lies within the tether. NOE data showed strong crosspeaks indicative of a type II or II' β-turn. NMR cannot readily distinguish between the two enantiomeric backbones in this case. Still, the CD spectrum of this compound was virtually superimposable onto that derived from *cyclo*(Ala-Gly-Aca) and verified the presence of a type II turn. This example demonstrated that a single stereocenter on the tether could strongly bias the conformation of the dipeptide to resemble a type II β-turn. Finally, analog **21** was synthesized to help determine the relative importance of dipeptide and linker configuration. The dipeptide stereochemistry continues to be the primary conformational determinant in these compounds. Any influence from the tether is secondary. Thus, a type I β-turn was the predominant conformation in **21**, presumably due to the L,L dipeptide.

In addition to the solution-phase studies, macrocycles **1**, **3**, and **4** were crystallized from methanol/CH_2Cl_2. X-ray analysis showed that the relevant dihedral angles all lay within 21° of the idealized values for type I and II β-turns (Fig. (**15**)). The positions of $Aca_{C=O}$ and Aca_{NH} in **3** and **4** indicated the presence of hydrogen bonding. These structures supported the earlier solution phase data indicating type II bends for **3** and **4** and mainly a type I bend for **1**. Although



1 **3** **4**

Important dihedral angles (degrees)

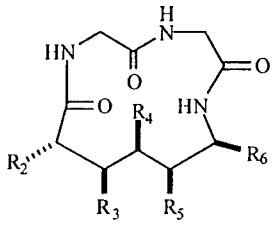
	ϕ_{i+1}	ψ_{i+1}	ϕ_{i+2}	ψ_{i+2}
Type I	-60	-30	-90	0
Type II	-60	120	80	0
1	-60.5	-42.8	-108.7	18.4
3	-73.3	108.6	84.7	8.3
4	-67.1	106.6	72.1	20.7

Fig. (15). X-ray structures of **1, 3,** and **4** and comparison of the pertinent dihedral angles.

the Ala-Gly dipeptide typically favored the βII turn, one of the four dimethyl-substituted Aca linkers was able to enhance the proportion of the observed βI turn.

Singly Substituted Aca

The finding that a single methyl group on Aca could direct a cyclized dipeptide into a distinct β-turn conformation suggested the study of other monosubstituted linkers. The next series of compounds included macrocycles consisting of Gly-Gly cyclized with an Aca linker containing a single alkyl group at each position of the carbon chain (Fig. (**16**)). As in **20**, Gly-Gly was used because it lacks side chains which influence the backbone geometry of the dipeptide. In this study, a benzyl group was chosen to aid in handling and visualization of the pseudopeptides and synthetic intermediates.

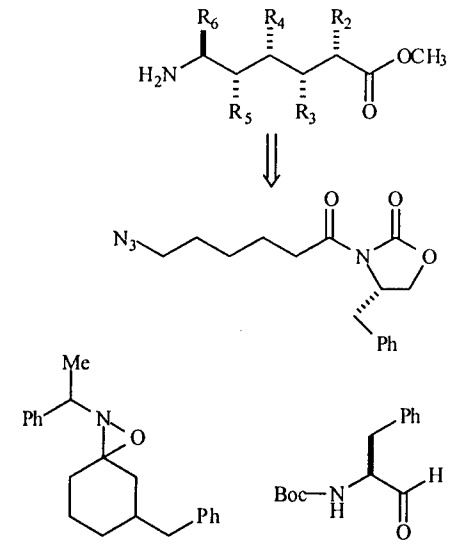


22: $R_2 = CH_2Ph$
23: $R_3 = CH_2Ph$
24: $R_4 = CH_2Ph$
25: $R_5 = CH_2Ph$
26: $R_6 = CH_2Ph$

R substituents not designated as an alkyl group are H

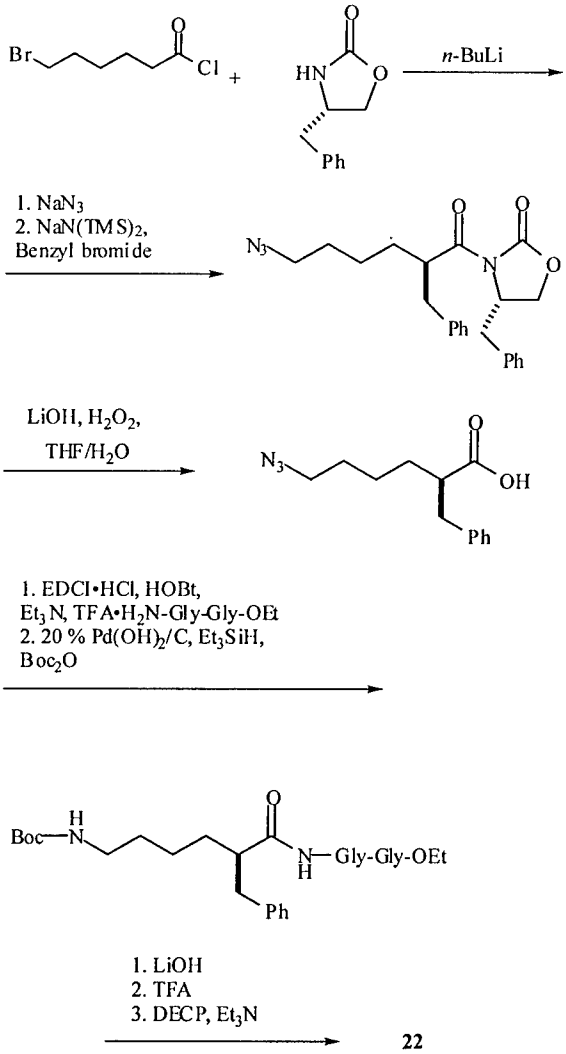
Fig. (16). A series of Aca-constrained dipeptides that incorporate a single benzyl substituent.

The linkers were synthesized in enantiomerically enriched form using one of three different methods, depending on the position of the substitution. The retrosynthetic analysis depicts the general routes to make each substituted Aca (Scheme 16). The synthesis of the C-2 substituted tether utilized an Evans alkylation to establish the C-2 stereocenter [74]. The 3-, 4-, and 5-benzyl Aca tethers were synthesized according to the oxaziridine protocol described above [66, 67]. The linker with the benzyl group in the C-6 position was derived from L-phenylalanal [75]. The overall syntheses leading to final products **22** and **26** differed from the routes used to reach **23-25**. The oxaziridine-mediated ring expansion produced the completed Aca derivatives as amino esters ready for incorporation into the tripeptide. Construction of the macrocycles comprising the C-2 and C-6 benzyl-substituted Aca tethers deviated from this procedure in that precursors to the final tethers were incorporated into the peptide, then modified in the final stages of the synthesis. The final amide bond formed during the ring closure was another difference in the macrocyclic syntheses.



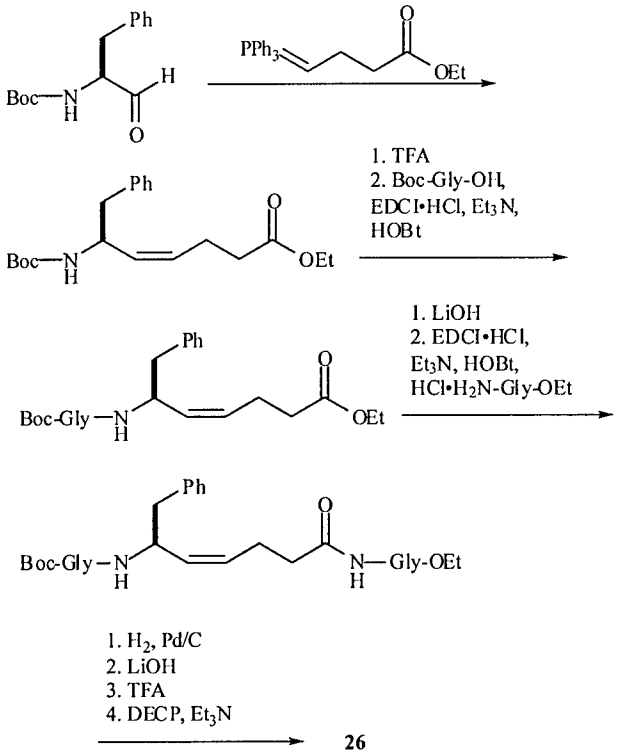
Scheme 16.

The key step in the synthesis of the C-2 benzyl linker was the establishment of the stereocenter via a stereospecific alkylation using the phenylalanine-derived oxazolidinone (Scheme 17) [74]. Coupling of 6-bromohexanoyl chloride to the auxiliary followed by nucleophilic displacement of the bromide with sodium azide afforded the alkylation substrate. Deprotonation, then alkylation with benzyl bromide, furnished 6-azido-2-benzyl-hexanoyloxazolidinone. Hydrolysis of the auxiliary [76] afforded an azido acid, which



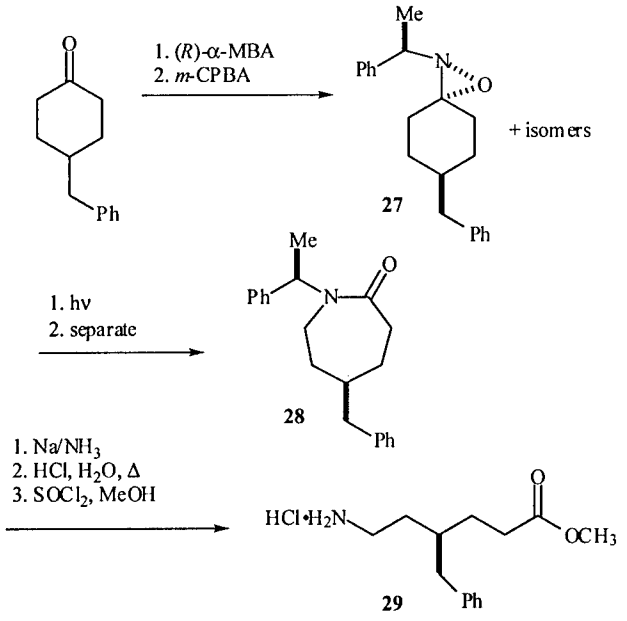
Scheme 17.

was used in the peptide construction. After the precursor was incorporated into the pseudotripeptide, the azide was reduced to the Boc-protected amine. *N*- and *C*-terminal deprotection was accomplished upon saponification of the ester and TFA removal of the Boc group. The cyclization occurred with neutralization of the TFA salt with triethylamine (Et_3N) and carboxylate activation with DECP at a 5 mM dilution to produce **22**.



Scheme 18.

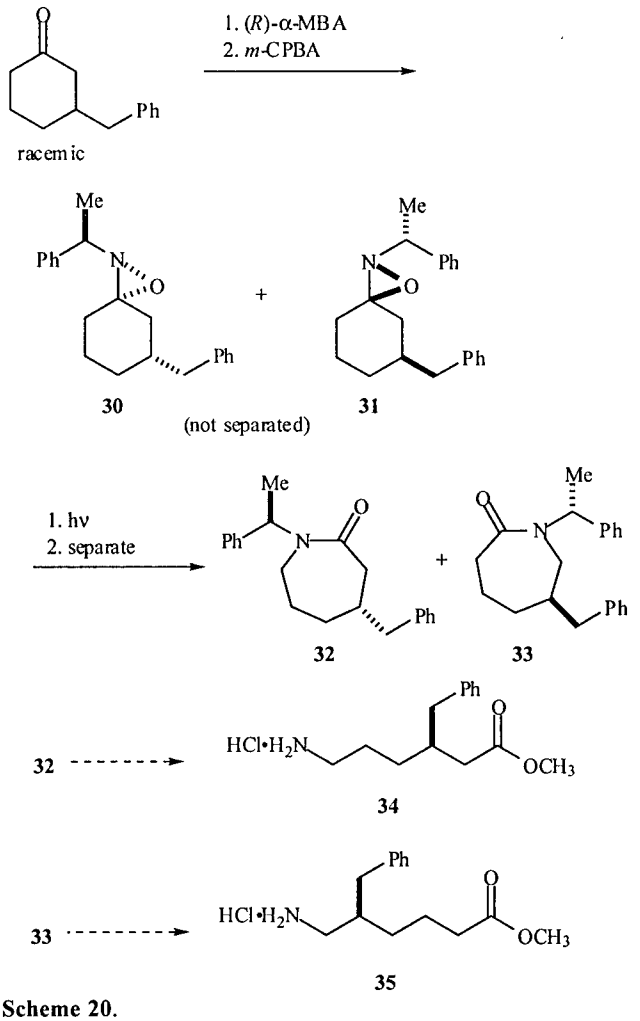
The Aca linker bearing C-6 substitution in **26** was derived from Boc-phenylalanal (Scheme 18). The aldehyde was subjected to Wittig conditions using the ylide of [3-(ethoxycarbonyl)propyl]triphenylphosphonium bromide [77] to extend the carbon backbone. The olefinic intermediate was incorporated into the acyclic peptide by attaching glycine residues at each end of the linker. Amino acid couplings followed standard procedure [78]. At this point, the olefin was reduced by hydrogenation. *N*- and *C*-terminal deprotection followed by cyclization yielded compound **26**.



Scheme 19.

The linker **29** was synthesized using the oxaziridine ring expansion sequence (Scheme 19) [66]. The starting ketone, 4-benzylcyclohexanone [79], was condensed with (*R*)-α-MBA to form the imine, which was treated with *m*-CPBA to form a mixture of oxaziridines, with **27** as the major diastereomer. The mixture was photolyzed to produce a mixture of lactams. Upon separation by column chromatography, the major lactam **28** was treated with Na/NH_3 to remove the phenethyl group. Acidic hydrolysis followed by an in situ esterification furnished **29** as the hydrochloride salt.

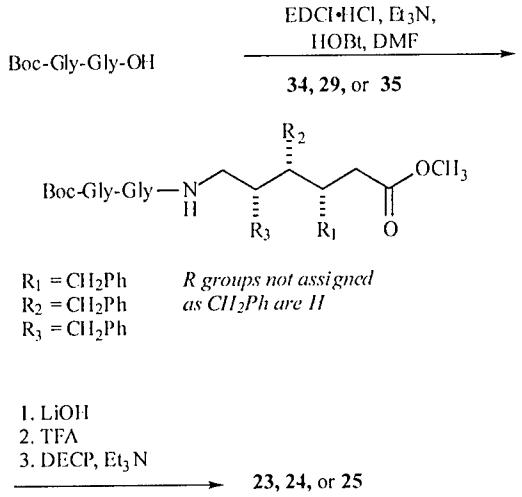
The synthesis of the 3- and 5-benzyl Aca moieties began with (±)-3-benzylcyclohexanone, which supplied two different linkers by undergoing ring expansion/resolution as described above in the syntheses of (3*R*)- and (3*S*)-methyl Aca [67]. The racemic ketone was converted to a mixture of oxaziridines that contained two major diastereomers, **30** and **31** (Scheme 20) [80]. The diastereomers were not separated



Scheme 20.

but photolyzed to form **32** and **33**, which were separated by column chromatography and further purified by crystallization from Et_2O/hexane. As before, treatment with Na/NH_3, acid hydrolysis, and esterification of each lactam furnished the hydrochloride salts of the monobenzyl linkers **34** and **35**.

The Aca tethers **34**, **29**, and **35** were coupled to Boc-Gly-Gly-OH, forming the corresponding triamides (Scheme 21) [78]. Hydrolysis of the ester and Boc removal provided the deprotected tripeptide, which was cyclized to form **23, 24,** and **25** using the conditions described earlier.



Scheme 21.

Due to difficulties encountered during the ring closure step, various conditions to promote cyclization were attempted, ultimately arriving at the conditions noted. After little success in closing the macrocycle by forming the Gly-Aca or Aca-Gly amide, as was done for compounds **22-25**, we decided to investigate the ring closure through cyclization at the less sterically encumbered Gly-Gly linkage. Different cyclization conditions were explored using the tripeptide, Gly-6-benzyl-Aca-Gly, as the model. The original conditions using DECP/$NaHCO_3$ which had been successful in the disubstituted Aca series, only produced low yields. Variations in the coupling agent, the base used to neutralize the TFA salt, and the solvent eventually led to the more successful combination of DECP and Et_3N in a mixture of DMF/toluene (1:1). Toluene was added to the mixture in the hope that greater hydrophobicity would aid in cyclization. It is worth noting that the cyclizations carried out on the tripeptides with the monosubstituted linkers and Gly-Gly gave lower yields than those with the 3,5-dimethyl Aca tethers and dipeptides containing more substitution.

Each macrocycle was subjected to NMR and CD analysis as described above. In general, the NMR data were consistent with either a type II or II' β-turn conformation based on crosspeaks observed between Gly-2_{NH} and one of the prochiral Gly-1_{α} protons as well as between Gly-2_{NH} and Aca_{NH}. The presence of the type I or I' bends seemed unlikely due to the lack of a crosspeak between Gly-1_{NH} and Gly-2_{NH}. Therefore, it was suspected that each of the macrocycles took on some form of βII or βII' turn as the predominant conformation.

CD helped to differentiate between type II and II' turns for each compound. The spectra of **22** and **23** resembled those of standard type II β-turns in that they both have a maximum near 200 nm and a minimum at 220 nm (Fig. (**17**)) [81]. The CD spectra for compounds **25** and **26** indicated a type II' β-turn, because the curves are nearly the mirror images of those data obtained for the former pair of macrocycles. Compound **24** did not resemble any of the reported turn types. The spectrum most closely resembled a type II curve, with an intense maximum at 200 nm and modest minimum near 230 nm. The NMR data also revealed some interactions that could be interpreted as arising from a βII turn; however, it would only be a small percentage of the solution structure.

X-ray structures obtained for compounds **24** and **26** are shown in Fig. (**18**). Consistent with the NMR and CD data, the crystal structure for **26** confirmed a type II' β-turn, placing the amide bond in the dipeptide nearly perpendicular to the macrocycle. The observed dihedral angles of the dipeptide unit were within 26° of the idealized βII' values. The structure also revealed a possible hydrogen bond between $Aca_{C=O}$ and Aca_{NH}.

Crystallographic analysis of **24** showed the presence of two distinct conformers, neither of which resembled a reported β-turn conformation. In each case, the backbone was positioned such that all of the amide protons pointed in one direction, causing the carbonyl oxygens to face the opposite direction. This orientation rules out the possibility of a

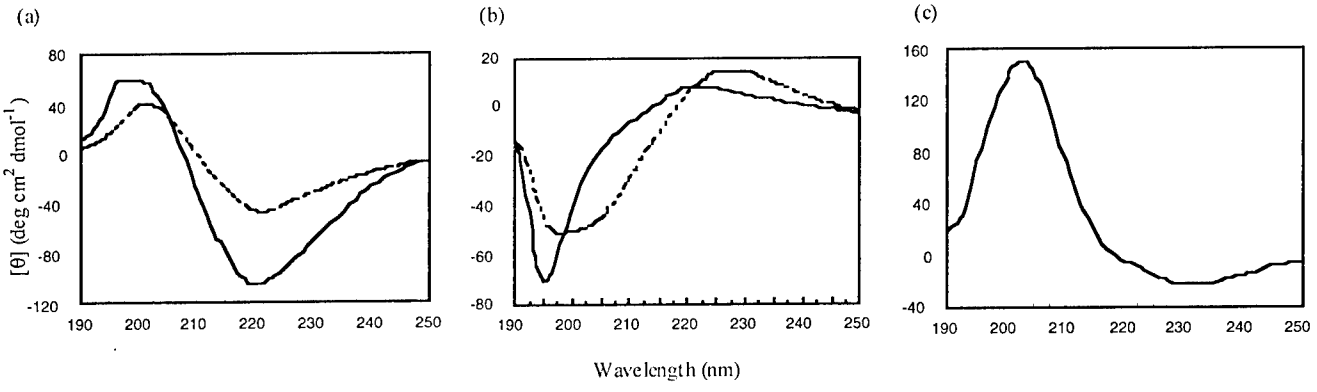


Fig. (17). CD spectra of macrocycles (a) **22** (solid line) and **23** (dotted line), (b) **25** (solid line) and **26** (dotted line), and (c) **24**.

hydrogen bond between $Aca_{C=O}$ and Aca_{NH}. Also, the 4-benzyl Aca was somewhat distorted from the backbone conformations seen in the previous X-ray structures. The tether was bent so as to place the benzyl group above the ring, in a pseudoaxial orientation, and to project the C-4 methine proton into the ring. It is likely that some of these observations may have resulted from crystal packing.

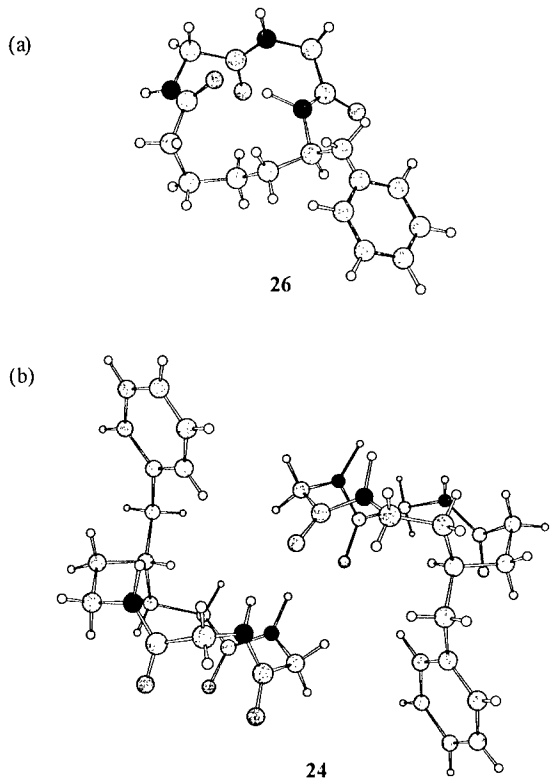


Fig. (18). X-ray crystallographic structures obtained for compounds (a) **26** and (b) **24**.

Other Applications for Aca and Aca-derived Peptides

Aca-constrained dipeptides have been used in studies to correlate conformation with cell permeability [82]. Two Aca-cyclized peptides and two similar *N*- and *C*-terminally capped acyclic peptides shown in Fig. (**19**) were subjected to membrane transport assays. The most significant result of this work was that changing a stereocenter in the cyclic peptide from L to D enhanced the rate of transport. This dependence of transport on stereochemistry was not evident in the acyclic controls. The difference in cell permeability was thought to originate from differential hydrogen-bonding patterns in the β-turn mimics, a supposition that was supported by chemical shift data.

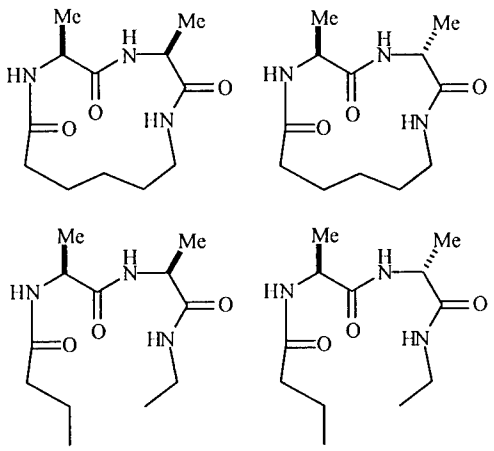


Fig. (19). The cyclic and acyclic peptides used in the cell permeability assays.

Table 2. Selected Dihedral Angles for 26 and 24 (deg)

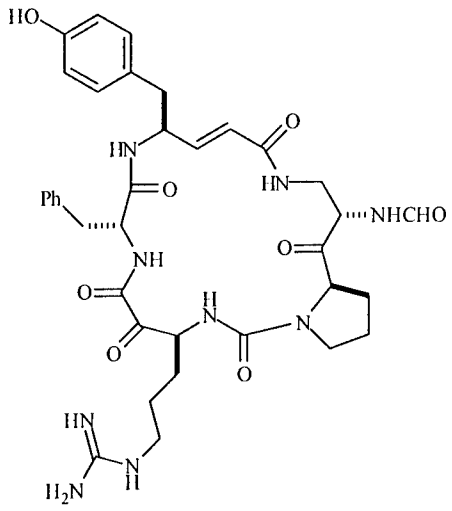
β-turn	ϕ_{i+1}	ψ_{i+1}	ϕ_{i+2}	ψ_{i+2}
Type I[a]	-60	-30	-90	0
Type II	-60	120	80	0
Type II'	60	-120	-80	0
26	71	-115	-67	-26
24 (conformer 1)	-54	53	-80	94
24 (conformer 2)	104	-77	68	38

[a] Idealized values [1]

Aca templates were also used to examine rates of deamidation of asparagine residues, a process of peptide degradation that produces either aspartic acid or isoaspartic acid [83]. Controlled hydrolysis of Aca-constrained dipeptides containing asparagine was carried out to correlate the position of asparagine in a β-turn with its rate of deamidation. It was noted that placement in the *i*+2 position compared to the *i*+1 position led to an increase in the half-life of the amino acid.

Aca, along with its five- and seven-carbon homologs has been incorporated into macrocyclic, tripeptide-based thrombin inhibitors (Fig. (**20**)). A series of analogs derived from cyclotheonamide A retained the necessary α-keto amide and D-Phe-Pro-Arg groups from the natural product but replaced the rest with a carbon tether [84]. All compounds were assayed to examine their ability to inhibit thrombin and trypsin proteases. In general, the constrained peptides demonstrated better activity than their acyclic counterparts. The most potent analog contained Aca and formed a 20-membered ring that positioned the tripeptide section in an extended conformation. This analog showed activity comparable to cyclotheonamide A.

Balaram and coworkers have investigated the orientation of α-helices connected via an Aca linker [85]. It was hoped that the flexible nature of Aca would allow the formation of a hydrogen bond between its amide proton and carbonyl oxygen and thus promote the formation of an α,α-antiparallel structure. A hydrophobic amino acid sequence, Val-Ala-Leu-Aib-Val-Ala-Leu (Aib = α-aminoisobutyric acid), was coupled to the amine and carboxylic acid groups on Aca. X-ray analysis of the dimer depicted an extended



Cyclotheonamide A

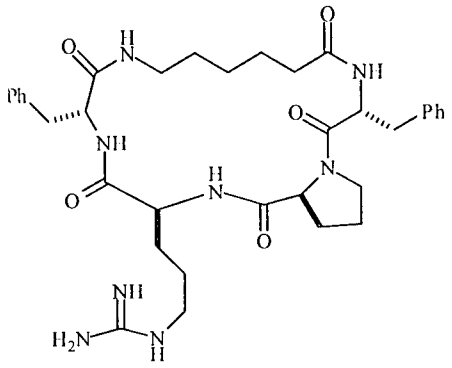


Fig. (20). Cyclotheonamide A and an analog incorporating Aca.

structure that placed the helices parallel to each other rather than the bent, antiparallel conformation expected (Fig. (**21**)). The observed structure was believed to be stabilized by head-to-toe hydrogen bonds between separate molecules forming a continuous helical column.

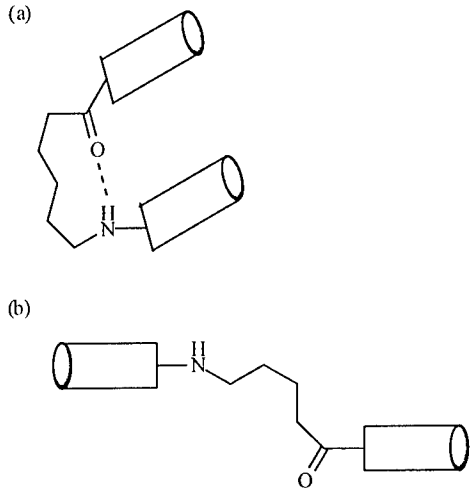


Fig. (21). Pictures of the Aca-linked α-helices in (a) the expected conformation and (b) the conformation deduced from the X-ray crystal structure.

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